The Interaction of *Mycobacterium tuberculosis* with Murine Dendritic Cells

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The interaction of microbes with dendritic cells (DCs) is likely to have an enormous impact on the initiation of the immune response against a pathogen. In this study, we compared the interaction of *Mycobacterium tuberculosis* with murine bone marrow derived DCs and macrophages *in vitro*. *M. tuberculosis* grew equally well within non-activated DCs and MØ. Activation of DCs and MØ with IFN-γ and LPS inhibited the growth of the intracellular bacteria in an NOS2-dependent fashion. However, while this activation enabled MØ to kill the intracellular bacteria, the *M. tuberculosis* bacilli within activated DCs were not killed. Thus, DC could restrict the growth of the intracellular mycobacteria, but were less efficient than macrophages at eliminating the infection. These results may have implications for priming immune responses to *M. tuberculosis*. In addition, they suggest that DCs may serve as a reservoir for *M. tuberculosis* in tissues, including lymph nodes and lungs.

Dendritic cells (DC) possess anti-microbial mechanisms that may play a role in *M. tuberculosis* infection. Activated DC and MØ produce a comparable amount of reactive nitrogen intermediates, as measured by nitrite production but our data indicate that nitric oxide production is not directly responsible for the differences in the ability of DC and MØ to kill *M. tuberculosis.* Activated DC have the capacity to produce greater quantities of reactive oxygen intermediate (ROI) than activated MØ. The higher levels of ROI in infected DC may affect the formation of different reactive nitrogen intermediate (RNI) species, such as peroxynitrite, and alter the lethal effect on *M. tuberculosis.* In addition DC may not able to acidify their phagosomal compartment,

where the bacilli reside, to the same degree as macrophages. This may contribute to the ineffectiveness of their anti-microbial compounds.

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Introduction

Epidemiology of *M. tuberculosis* infection

The disease tuberculosis is caused by the bacterium *Mycobacterium tuberculosis,* which is a highly infectious organism transmitted via aerosol. In 1999 the World Health Organization (WHO) estimated that *M. tuberculosis* caused 8 million new cases of tuberculosis and 1.8 million deaths (1). Until the early 1800s tuberculosis was one of the leading causes of death in Europe and the Americas, however improved living conditions as well as the start of successful chemotherapy led to a decrease in mortality rates (1). This disease persisted in developing countries where it is still responsible for 25% of the avoidable deaths among adults and a leading cause of death among young women (1). A recent epidemiological study estimated that approximately 1.8 billion persons, which is one third of the world's population, are infected with *M. tuberculosis* (1). This number will increase with the expanding population, inadequate treatment and health care, civil disturbances, and importantly, the human immunodeficiency virus (HIV) pandemic.

Tuberculosis is most prevalent in developing countries that harbor both 95% of the world's *M. tuberculosis* infection (2) and 90% of the global HIV infections. HIV causes acquired immunodeficiency syndrome (AIDS) and has emerged as one of the greatest risk factors for the development of active tuberculosis in persons co-infected with both *M. tuberculosis* and HIV (2) (3). AIDS increases the risk of progressing to tuberculosis that results in death (4). Indeed, tuberculosis was reported to be the leading cause of death among HIV patients in 1999 (1). *M. tuberculosis* infection may accelerate HIV replication; viral recovery from lungs of HIV-infected patients increased following the development of tuberculosis and returned to baseline following successful anti-tuberculosis chemotherapy (5). The correlation

with *M. tuberculosis* infection and HIV replication rate may result from immune activation accompanying tuberculosis (6). HIV and tuberculosis together result in much socioeconomic hardships for those who are afflicted and this contributes to a global financial burden (1). The World Health Organization uses a measure of disease burden called a DALY, Disability-Adjusted Life Year. DALYs express the years lived with a disability, adjusted for by the severity, and the years of lost life due to premature death (1). HIV and tuberculosis together accounted for 7% of all DALYs in 1998.

If diagnosed, tuberculosis can be treated successfully with a combination of several antibiotics. However, the antibiotic regimen is long- six to twelve months- and the high level of non-compliance among patients encourages the development of multi-drug resistant strains of *M. tuberculosis* (MDR-Tb). In New York City, 33% of tuberculosis strains recovered in 1992 were resistant to one drug and 19% were resistant to two or more drugs (7). In Asia and Eastern European countries there has been an increase in MDR-Tb (1), (8), (9). The World Health Organization developed the Directly Observed Treatment- Short-course (DOTS) strategy in the early 1990s in the Western Pacific to help improve treatment. Under DOTS, patients take the antibiotics under direct supervision to help reduce non-compliance and subsequent drug-resistant strains. DOTs is now in use in 28 countries and treats 35% of all cases (1). Recently this strategy is being used in New York City where non-compliance, as well as MDR-Tb, is high. When properly administered, DOTS has achieved up to a 90% cure rate (1) but many developing countries lack the resources to properly implement it. Insufficient treatment programs have resulted in drug-resistant rates in excess of 30% in some developing nations (10). For instance, the prison system in Russia gave prisoners partial antibiotic treatments for tuberculosis, which resulted in a very high number of drug resistant strains in the prison system (40%) and

subsequently a doubling (3-6%) of MDR-Tb in civilian Russia (11). In light of the complex nature of *M. tuberculosis* and the increasing number of HIV infected people, an increase of MDR-Tb strains and cases, a rapid expansion of third world's population, increasing elderly population and numerous cases of reactivation, this disease demands a considerable amount of attention, both medical and research, now and in the future.

Currently, no effective vaccine is available. A vaccine made from an attenuated strain of *Mycobacterium bovis,* bacillus Calmette-Guérin (BCG), remains the sole vaccine against tuberculosis but its efficacy is variable and has been estimated to range from 0 to 80% effective (12). The only way to eliminate the spread of *M. tuberculosis* is to develop a protective and reliable vaccine. Recently, DNA vaccines based on the antigen 85 complex (13) or the 65 kD heat shock protein (hsp) of *M. tuberculosis* (14) have provided partial protection against a challenge with virulent *M. tuberculosis* in mice. DC infected with *BCG*, or *M. tuberculosis* have been shown to confer protection to challenge with *M. tuberculosis* in a murine model (15). A greater understanding of the immune response is necessary in order to develop a vaccine that will confer complete protection from infection and, hopefully, from reactivation of a pre-existing infection.

Characteristics of Mycobacterium tuberculosis

Mycobacteria have a straight or curved rod morphology and exist either singly or in clusters. *Mycobacterium tuberculosis* is a facultative intracellular aerobic pathogen that resides primarily in macrophages of many mammalian hosts such as human and cattle. *M. tuberculosis* is a particularly slow growing bacterium and divides once every 18-24 hours. Therefore it takes between 18-21 days to view visible colonies on solid medium, which slows down research of this

organism. The cell wall of *M. tuberculosis* has an unusually high lipid content and has many components which are unique to mycobacteria, such as cord factor which causes the bacilli to clump in cell culture and again impose a problem in experimental infections (12).

The cellular envelope of *M. tuberculosis* consists of a plasma membrane and a highly unusual cell wall. The plasma membrane consists of a classical bilayer structure. The elaborate distinctive features of the mycobacterial cell walls include the lipoarabinomannan (LAM) (16), lipomannan, mycolyl-arabinogalactan, phosphatidyl-myo-inositol mannoside, sulfatide, cord factor, and other acylated trehaloses, phenolic glycolipids, lipoligosaccharides, and other attenuated lipids (12). Many of these have been shown to be involved in the virulence and pathogenesis of this bacillus (12). LAM, a predominant component of the cell wall, is a virulence factor for *M. tuberculosis*, and activates macrophages and scavenges reactive oxygen intermediates (17). When LAM is mannosylated there is a loss of virulence (18). The most common characteristic of the cell wall is peptidolglycan that is surrounded by covalently linked phospohdiester bonded arabinogalactan with mycolic acids attached to it. The arabinogalactan and mycolic acid complex is a strong immunogen (19).

An important feature of the mycobacterial cell wall is the integrity of the outer matrix mycolyl-arabinogalactan and the integrity of the capsular lipids (20) that keeps the cell wall very rigid, highly organized, and very densely packed with lipids. Due to the unique composition and high lipid density of the cell wall, mycobacteria can be detected by an acid fast staining technique. In this technique the bacteria retain the primary dye after decolorization with an acid alcohol, carbol fushsin, due to the high lipid content of the cell wall and mycobacteria are referred to as acid fast (12). It has been hypothesized that this unique cell wall ensures the survival and replication of the bacteria *in vivo*. The cell wall structures may help evade

complement-mediated lysis, prevent anti-microbial mechanisms within the macrophages, and downregulate a successful immune response (12)

Mycobacterium tuberculosis Virulence and Disease Pathogenesis

The development of tuberculosis from infection may be viewed as a series of battles between the host defenses and the bacilli (12). The development of the disease tuberculosis can occur decades after infection and the bacterium is very complex which makes it very hard for the host to eliminate and sometimes control *M. tuberculosis*. *M. tuberculosis* infection can result from the inhalation of as few as 10-50 bacilli that are swallowed up by alveolar macrophages. *M. tuberculosis* can gain entry into human macrophages, their host cell, by utilizing a wide array of receptors depending on whether or not the bacilli are opsonized with complement, antibodies or surfactant-A (reviewed in (21)). Putative receptors include complement receptors-1, 3 and 4, the macrophage mannose receptor, class A scavenger receptors, CD-14, Fcγ receptors and surfactant-A receptors (reviewed in (21)). It may be possible that all the invading bacilli are killed immediately within the macrophages, thereby preventing infection. There is no direct evidence that this scenario occurs, although it is generally believed that certain people are resistant to infection; these persons, who are often health care workers, are repeatedly exposed to *M. tuberculosis* but do not convert to PPD+ or develop tuberculosis. However, it is unclear how common this is. If the bacilli do survive within the alveolar macrophages, they replicate and invade the lymphatics and bloodstream, spreading throughout the body, often seeding the apical lung segments and may lead to disease. Other organs also may become infected resulting in extrapulmonary tuberculosis, most commonly arising in the lymphatics, pleura, bones, genitourinary tract, and meninges of the central nervous system (CNS). Both human (22) and murine (23) dendritic cells can internalize *M. tuberculosis* and these migratory cells may play a

role in dissemination of the bacteria to lymphoid organs. An infected person may control the initial infection, but it is believed that the infection is generally not eliminated. However, control of the infection with no symptoms is the most common outcome of infection, and this is referred to as latent tuberculosis. A person with latent tuberculosis may reactivate, even after many years, under a variety of conditions such as HIV, malnutrition, age, or steroid use. However, more commonly, the infected person never develops disease. It is not fully understood how or why reactivation occurs or whether the bacilli are dormant or proliferating during this period (12).

Approximately 5-10% of primary infections progress to active pulmonary tuberculosis; in which a lesion will generally be visible upon chest radiograph. In tuberculosis, the tubercle bacilli induce the formation of characteristic granulomas in the lungs or other infected organ. These lesions consist of central macrophages that become enlarged or "epithelioid" surrounded by lymphocytic cells and fibrocytes. In humans, these epithelioid macrophages may fuse to form multinucleated giant cells. As the disease progresses, the granulomas increase in size, become necrotic and create pathology of the affected organ. In pulmonary tuberculosis, the patient becomes highly contagious as lungs expel debris containing bacilli in tiny droplets whenever the patient coughs, sneezes, or even speaks. These granulomas are a delicate balance between the struggle for the host's immune response to control the bacterial growth and the bacteria's desire to persist in this harsh environment. Ironically, the damage sustained to the host results at least in part from the products of the immune system (12).

Macrophage Effector Functions

It has long been known that macrophages possess anti-microbial functions (24), (25), (26). Mononuclear phagocytes, such as macrophages, constitute a potent anti-microbial component of the cell-mediated immune response (12). The best characterized effector

mechanisms include phagosome-lysosome fusion, the reactive oxygen intermediate (ROI) burst, and the production of reactive nitrogen intermediates (RNI) by an L-arginine-dependent pathway (12). *M. tuberculosis* is equipped with several features that increase its chances of survival within macrophages.

Phagolysosome-Fusion

Once ingested by a phagocytic cell an intracellular pathogen is sequestered away in a membrane bound phagosome unless they have mechanisms to escape out of the phagosome and into the cytoplasm (27), where they will be processed for MHC class I presentation. It is an advantage for a microbe to hide in the phagosome, however the organism must be able to either prevent lysosomal fusion or acidification. The phagosome progresses through the endocytic pathway, characterized by the acquisition of small GTPase proteins, Rabs, which regulate endosomal membrane trafficking (28), until it fuses with the lysosome. The lysosome is a very complex organelle that contains hydrolytic enzymes that can degrade a wide variety of macromolecules, including microbes. These enzymes function best at an acidic pH and therefore the lysosome maintains a pH of 4.5-5.5 (29). This environment is maintained by ATPase proton pumps in the vacuole (30). The fusion of the phagosome to the lysosome is a dynamic process since it involves the maturation of the phagosome. *M. tuberculosis* and *M. avium* can inhibit both phagosome acidification and phagosome-lysosome fusion (30), (31). If *M. tuberculosis* is within these conditions of the phagosome-lysosome it can be killed (32). Studies have shown that *M. tuberculosis* arrests the phagosome maturation at the early endosome stage, which expresses Rab 5, and this results in the phagosome being unable to proceed to fuse with the lysosome (33). *M. tuberculosis*- or *M. avium*-infected cells have been shown to exclude the ATPase proton pump from the phagosome, which would also prevent acidification (30), (31). Although *M. avium*

appears to have many similarities to *M. tuberculosis* in the way that it resides within the phagosome, there are many differences between these bacteria, which result in altered disease pathogenesis. *M. avium* only can cause disease in immunocompromised people.

Recent data from studies using BCG suggest that this inhibition may result from the internalization of cholesterol into the macrophage that occurs when mycobacteria enter through cholesterol-rich domains of the macrophage cell membrane (34). This cholesterol co-localizes with the mycobacteria in the phagosome and may cause retention by the phagosome of tryptophan-aspartate-containing coat protein (TACO), preventing phagosome-lysosome fusion (34), (35). *In vitro* studies have suggested that *M. tuberculosis* can generate copious amounts of ammonia in cultures which could be responsible for preventing phagosome-lysosome fusion as it affects lysosome trafficking and alkalinizes the intracellular environment of the lysosome (36). Two enzymes which contribute to ammonia metabolism have been investigated, urease and glutamine synthetase. Glutamine synthetase has actually been shown to be released from monocytes during infection with *M. tuberculosis*. It is released from virulent species in cultures, such as *M. tuberculosis* and *M. bovis* but not from non-pathogenic strains such as *M. smegmatis* (37), (38). Overall there is substantial evidence that *M. tuberculosis* can block phagosome-lysosome fusion so that it can reside within the macrophage. However, upon activation there is some phagosome-lysosome fusion resulting in bacterial death.

Reactive Oxygen Intermediates

The production of reactive oxygen intermediates (ROI), or the respiratory burst, plays a significant role against microbes, such as *Salmonella* species (39). The importance of ROI is demonstrated by the frequent uncontrolled infections seen in chronic granulomatous patients. These patients have a defect in NADPH oxidase complex, a critical respiratory burst enzyme

complex, therefore they cannot produce ROI (40, 41). The role that ROI play in *M. tuberculosis* infection is controversial. ROI were shown to kill *M. microti,* which at first led researchers to believe they may kill *M. tuberculosis*. However, evidence supported quite the opposite. A major mycobacterial cell wall component, lipoarabinomannan (LAM), was effective at scavenging ROI generated by the respiratory burst of infected macrophages (18). It was also shown that ROI can inhibit protein kinase C which downregulates the respiratory burst (18). Tubercle bacilli also produce both superoxide dismutase (42) and catalase (43) that may interfere with toxic oxygen radical production. Other mycobacterial components such as sulfatides and phenolglycolipid I (PGL-1) have also been suggested to interfere with ROI-dependent anti-microbial mechanisms within macrophages (18). *M. tuberculosis* could also evade the respiratory burst by avoiding binding to macrophage receptors, such as Fc receptors, but instead use complement receptors. *M. tuberculosis* has been shown to use CR3 to enter into macrophages (44), (45). A macrophage line which is deficient in ROI production was shown to be able to control the infection the same as wild-type controls suggesting that ROI are not required to control infection *in vitro*(46).

However, other studies support a role for ROI in control of *M. tuberculosis* infection. Hydrogen peroxide generated by cytokine-activated macrophages was shown to have mycobactericidal effects (47). Infection of phox-/- mice, which are deficient in NADPH oxidase complex, were initially more susceptible to *M. tuberculosis* infection (40), (41). Humans with chronic granulomatous disease are more susceptible to tuberculosis (48). In addition, ROI may form other compounds, such as peroxynitrite or peroxynitrous acid, which have been shown to kill some mycobacterial species (49).

Reactive Nitrogen Intermediates

Phagocytic cells, such as macrophages and more recently DC, have been shown to produce nitric oxide (NO) and other reactive nitrogen intermediates (RNI) via the NOS2 enzyme using L-arginine as a substrate. NOS2 is induced by interferon-γ (IFN-γ) and a second signal such as tumor necrosis factor (TNF-α) or bacterial products such as LPS or LAM (50), (51), (52), (23), (53), (54), (55). NO is highly reactive, has a very short life, and can quickly be oxidized to form nitrite, NO_2^-, a more stable compound. In an acidic environment, such as the phagolysosome, chemical reactions can generate many RNI species such NO_2, HNO_2, N_2O_3, and in conjunction with ROI can form peroxynitrite ($ONOO^-$) and peroxynitrous acid (HOONO) (56).

Nitric oxide production within macrophages has major anti-microbial mechanisms (52), (57), (50), (51). RNI can inflict damage to the bacterium by modifying DNA, proteins, and lipids (51), (57). In the murine system, toxic nitrogen compounds have been shown to play a role in protection in both an acute and a chronic *M. tuberculosis* infection (58), (59). NOS2-derived RNI are the only clearly demonstrated mycobacterial effector function in macrophages (60), (46), (61), (62), (23). Activated (IFN-γ + LPS or TNF-α) macrophages (cell lines and murine bone-marrow derived macrophages) can routinely kill 50% of intracellular *M. tuberculosis* which is not enough to clear infection (46), (23), (62), (18). However, macrophages derived from NOS2-deficient mice do not kill *M. tuberculosis*, nor do wild type bone-marrow macrophages which have been treated with an NOS2 inhibitor such as aminoguanidine (AG) or N^G-monomethyl-L-arginine (NMMA) (58), (46). Mice given AG or NMMA to block RNI synthesis succumbed to infection with *M. tuberculosis* in acute and chronic infection models (46) (63). NOS2 gene-disrupted mice died after infection with *M. tuberculosis* (64) (65). These

studies clearly demonstrate that NOS2 is essential to control of *M. tuberculosis* infection in mice *in vivo*. There are also data suggesting a role of NOS2 in humans. A high level of NOS2 expression was seen in bronchial lavage macrophages from patient's with active tuberculosis (66), (67). In one study patients with tuberculosis exhaled more nitric oxide (67). More macrophages obtained from tuberculosis patients expressed NOS2 as compared to normal non-diseased people (66). Despite the toxicity of these RNI compounds, only 50% of the intracellular *M. tuberculosis* is killed in murine derived macrophages and cell lines. It is yet to be determined if and how much *M. tuberculosis* is killed within human macrophages. Thus, while this is a very important mechanism for protection, other critical factors which remain to be identified.

Peroxynitrite

Peroxynitrite, as described above, is a strong oxidant produced by macrophages and neutrophils (68). Peroxynitrite is highly bactericidal for many bacteria including *Escherichia coli*, and *Salmonella* (69), (39). Peroxynitrite has a longer half-life and is better at killing in a low pH, such as found in the phagolysosome (56). Peroxynitrite is formed from NO and the ROI superanion (0_2^-), which are produced simultaneously in macrophages (56). The equation for this reaction is $NO^{\cdot} + {}^{\cdot}0_2^- = ONNO^-$. It was believed that peroxynitrite production may contribute to the RNI-dependent killing of *M. tuberculosis.* However, one study demonstrated that *M. tuberculosis* is actually highly resistant to very large quantities of peroxynitrite (49). In this study, clinical strains as well as laboratory strains of virulent *M. tuberculosis* were highly resistant to peroxynitrite in a cell-free system, whereas non-pathogenic and non-virulent species or strains, such as *M. smegmatis* and BCG (avirulent *M. bovis*) were susceptible (49). All of these species and strains were susceptible to nitric oxide and nitride oxide killing, which

suggested that virulent strains of mycobacteria have evolved to avoid peroxynitrite killing. These bacilli have recently been reported to exhibit peroxynitrite reductase activity that may allow it to convert toxic peroxynitrite to nitrate (70).

Toll-Like- Receptors

The discovery of Toll-Like Receptors (TLR) in vertebrates has provided a link between innate immunity and adaptive immunity (71), (72),(73), (74), (75), (76), (77), and (78). Toll was first identified as being responsible for protecting Drosophila against fungal infections (79). Subsequently their link to adaptive immunity was shown. Toll receptors provide a signal for NF-κB-mediated cytokine cascades involved in anti-microbial mechanisms (80) and TLR likely play a role in regulating *M. tuberculosis* infections. The immunogenic properties of 19 k-Da mycobacterial lipoprotein were shown to be dependent on TLR-2 (81) (157). *M. tuberculosis* was shown to activate either TLR-2 or TLR-4 *in vitro* and in a CD14 independent manner (82). Importantly many of the immunogenic compounds such as LAM and mycolylarabinogalactan-peptidoglycan complex, and various mycobacterial lipids have been shown to signal through either TLR2, TLR4, or both (82). Recently, Modlin, et. al. reported that *M. tuberculosis* induced nitric oxide production via signaling through TLR (83). NO production is an important anti-microbial mechanism for *M. tuberculosis* infection. It will be of interest to determine whether and how this contributes to not only macrophage activation, but DC activation as well since DC express TLR, but not substantial levels of CD14 (84). Lipopeptides, which include bacteria products, were shown to stimulate dendritic cell maturation through TLR2 (85). As well it may be speculated that difference in the levels of TLR on DC versus macrophages may contribute to different levels of cytokine expression.

Immune Responses to *Mycobacterium tuberculosis*

A murine mouse model has been used to investigate both the acute and latent infection of *M. tuberculosis* since Koch first discovered the bacteria (12). This system has demonstrated that cell-mediated immunity that is dependent upon the production of inflammatory cytokines, is important for protection from disease. IFN-γ from T cells activate macrophages to produce RNI that can kill *M. tuberculosis*. Many of these components have been shown to be necessary for resistance in humans as well. Ironically, the same immune responses that are critical for protection are also partially responsible for the disease-associated pathology and longevity of infection. The following section details the various components of the immune response to *M. tuberculosis.*

Interleukin 12

To control *M. tuberculosis* infection, a Type 1 T cell response must be generated, as in the case of most intracellular infections. Macrophage and DCs produce IL-12 in response to *M. tuberculosis* infection leading to the development of a TH1 response and IFN-γ production (23), (86), (14), (87), (88). Exogenous administration of therapeutic IL-12 and the finding of susceptibility in of IL-12p40 gene deficient mice strongly support an important role for this cytokine in the protective immune response against *M. tuberculosis* (89),(90). In absence of IL-12 production, lower levels of IFN-γ production were generated upon bacterial challenge; which probably contributed to the susceptibility of the IL-12 deficient mice to the infection. Human cases of disseminated BCG or *M. avium* infections have been linked to mutations in genes encoding the IL-12 receptor. These patients are deficient in T cell production of IFN-γ

production (91). Vaccination in conjunction with IL-12 DNA has been reported to reduce the bacterial load in *M. tuberculosis* in chronically infected mice (92).

Interferon−γ

IFN-γ is a critical cytokine involved in the control of *M. tuberculosis* infections. IFN-γ is produced by $CD4^+$ and $CD8^+$ T cells in *M. tuberculosis* infection. NK cells are another source of IFN-γ, but they have not been shown to directly to produce this cytokine in response to *M. tuberculosis* (93), (94), (95), (96), (97). Mice with a genetic deficiency for IFN-γ are the most susceptible to infection with virulent *M. tuberculosis* and have a mean survival time of only 14 days (98), (99). Notably there was also less NOS2 production in these mice indicating that macrophage activation was defective, contributing to the susceptibility of IFN-γ knockout mice. However, this is not the only critical factor since NOS2 gene-disrupted mice have a mean survival time which is twice as long as IFN-γ-/- mice (98), (50). The importance of this cytokine is also confirmed in humans who have a mutation in their IFN-γ receptor and this was associated with a heightened susceptibility to mycobacterial infections (100),(101). IFN-γ have been detected in the granulomas of patients and their T cells have been shown to produce IFN-γ in response to challenge with *M. tuberculosis* (102), (103). Although this is evidence for the direct importance of IFN-γ in human, the activation of human macrophages with simple addition of IFN-γ is not sufficient to kill *M. tuberculosis in vitro* (104). However, macrophages harvested from tuberculosis patient express NOS2 indicating that they have been activated *in vivo* (66), (67). This suggests that *in vitro* systems have not been able to sufficiently mimic what happens *in vivo*. In essence, we have not identified how to properly activate human macrophages to produce RNI. Some studies have shown that IFN-γ is depressed in the serum of patients with

tuberculosis. It has even been suggested that *M. tuberculosis* or its components inhibit macrophages to respond to IFN-γ by inhibiting STAT1-dependant signaling (105).

Tumor Necrosis Factor α

TNF-α is required for the control of an acute *M. tuberculosis* infection. TNF-α is secreted from DC and macrophages in response to *M. tuberculosis* infection (88), (86), (23), (14). Mice deficient in TNF-α or the 55-kDa TNF receptor succumb more quickly to *M. tuberculosis* infection (106), (107). Again, these mice have a high bacterial burden compared to wild-type controls. TNF-α acts as a necessary second signal (in addition to IFN-γ) to activate macrophages to produce RNI (108), (109), (61). NOS2 expression is delayed in the TNFRp55-/- mice but not in TNF-α-/- mice (106). Overall, the role of TNF-α in a *M. tuberculosis* infection is complex since it appears to contributes to both protection and pathology (110).

Interleukin-10:

IL-10 is an anti-inflammatory cytokine, which is produced by macrophages and T cells, leading to downregulation of IL-12 and consequently IFN-γ secretion by T cell and macrophages activation. Inhibition of IL-10 can help reverse the suppression of T cell proliferation *in vitro* by macrophages isolated from human tuberculosis patients (111). IL-10 has been shown to directly inhibit APC functions and T cell responses to mycobacteria infection (112). Although these results suggest IL-10 can counteract the ability of IFN-α to activate of macrophages, IL-10-/- knock-out mice were not shown to be more resistant to infection with *M. tuberculosis* (113). The role for IL-10 in *M. tuberculosis* infection is still not very well understood. Murine DC infected with *M. tuberculosis* do not produce IL-10 and therefore the deactivation of DC may come from

other cytokines, such as TGF-β (23). In other systems, TH1-polarized DC in the presence of IL-10 or TGF-β induced TH-2 like responses (84).

Cell Mediated Immunity:

Protection against *M. tuberculosis* is dependent on the development of a TH1 response. Since this bacteria resides intracellularly in macrophages and DC, an antibody response is not helpful, but T cell effector functions and macrophage activation can facilitate the elimination of the bacteria from within the cell. Both $CD4^+$ and $CD8^+$ T cells have been shown to play important roles in regulating the infection.

$CD4^+$ T cells:

$CD4^+$ cells are the most prevalent in the CMI toward *M. tuberculosis* and this is not surprising, since mycobacterial antigens can be processed intracellularly and then be presented by MHC II molecules. The necessity for $CD4^+$ T cells in bacterial resistance has been demonstrated in the murine system using antibody depletion (114), adoptive transfer systems (115), (116), and mice deficient in MHC II and $CD4^+$ (15), (117). The mean survival time for CD4-/- mice infected with *M. tuberculosis* is 60 days and the MST for MHC II-/- is 40 days whereas wild type mice will not die from the infection. There is a heavy recruitment of $CD4^+$ T cells to the lungs after IV infection with *M. tuberculosis* (116). In humans, the necessity for $CD4^+$ T cells to help control infection is shown by the rapid acceleration of tuberculosis in HIV+ patients who have a loss of $CD4^+$cells (118). The predominant role for $CD4^+$ T cells in protection is believed to be their production of IFN–γ, which will help activate macrophages to kill *M. tuberculosis*. However, this may not be the sole function for $CD4^+$ T cells in these infections. For instance $CD8^+$ T cells have been shown to contribute substantial amounts of IFN-

γ in CD4-/- cells (117), however this did not prevent the mice from ultimately succumbing to the infection. Also in this study NOS2 levels returned to normal after several weeks. These data demonstrate an IFN-γ and NOS2 independent mechanism that plays a dominant role in protection from disease. This has led to the investigation of other pathways by which $CD4^+$ T cells may protect against infection. Possible pathways which are still under active investigation include the perforin and granulysin, FAS-L or TNF-α lytic and apoptosis pathways (119), the interaction of CD40-CD40L between $CD4^+$ T cells and to enhance APC function (120), (121), (122), as well the ability of $CD4^+$ T cells to provide help to $CD8^+$ cells that may mediate a cytolytic response (Serbina, et. al. Journal of Immunology, in press).

$CD8^+$ T cells

The contribution of $CD8^+$ T cells in infection has been debated for a long time. At the crux of this debate is whether *M. tuberculosis* is ever found outside the phagosome (i.e. cytoplasm) where it could be processed for MHC I presentation. Although it still has not been exhaustively determined as to how mycobacterial antigens are presented to the immune system, $CD8^+$ T cells that are specific for mycobacterial antigens have been isolated from both infected humans and mice. $CD8^+$ T cells have also been shown to accumulate in the lungs after *M. tuberculosis* infection in mice. These cells can produce IFN-γ and lyse infected macrophages (97), (123), (93), (124). Earlier studies with mice deficient in the genes for β2-microglobulin, TAP, MHC I, and $CD8^+$ have shown that these mice exhibit heightened susceptibility to *M. tuberculosis* infection, and therefore $CD8^+$ T cells must contribute to protection (125), (126), (127). There have been recent data that MHC-I restricted $CD8^+$ T cells can recognize several antigens including some heat shock proteins (128). H_2-M_3-restricted $CD8^+$ T cells specific for

N-formyl peptides of *M. tuberculosis* have been reported to produce IFN-γ and lyse infected macrophages (129). CD1 molecules are structurally similar to MHC class I molecules but have differences in their binding groove which allow for the binding and presentation of lipids and glycolipids instead of protein antigens (130). Therefore they are believed to serve as important presentation complexes for mycobacterial antigens, and indeed, this has been shown for many mycobacterial lipids including LAM (131). However the actual role of T cell in response to these complexes in infection has been unclear.

Mechanisms by which mycobacteria could be processed for MHC I presentation have been recently suggested. Mice without CD1d were not more susceptible to infection (126), (127). Since DC have recently been shown to be important APC in a *M. tuberculosis* infection and they express CD1, this may be relevant to protection. It has been reported that mycobacterial bacilli were found outside of the phagosome several days post-infection (132). Other evidence shows that mycobacteria can make a pore in the phagosomal membrane that envelops them, allowing for the mycobacterial antigens to gain access to the cytoplasm (133). Increasing evidence supports the role for $CD8^+$ T cell in preventing tuberculosis infections but a debate remains as to the exact mechanisms by which this occurs.

Indeed the roles of both $CD4^+$ and $CD8^+$ may be multi-faceted and the two cell types likely collaborate in the generation of protective cellular immunity. Therefore understanding how to activate both T cell types in a vaccine is probably crucial to generating protection and long term memory.

Dendritic Cells

The high degree of specificity displayed by T and B lymphocytes in recognition of antigens is achieved with the help of antigen presenting cells (APCs), particularly, DC. DC are bone-marrow derived cells of the hematopoietic lineage which are potent APC. DCs have both an immature and mature stage. Immature DC are highly phagocytic and serve as scavengers for debris and pathogens. After stimulation with cyokines, T cell interaction or bacterial stimulus, the DC reach a mature stage, in which they dramatically enhance their antigen presenting functions. In the mature stage, DC upregulate not only important cell surface markers for T cell interaction, but adhesion molecules, and chemokine receptors. These allow DC to migrate from the site of infection to the draining lymph nodes in order to stimulate naïve T cells. DC are scattered throughout the body and are observed at various portals for infection (84). In the lung, DC are found in the interstitium and the airways (134).

Historically it was believed that alveolar macrophages were the dominant APC in the lung, however resident macrophages are not very migratory from the site of infection. Alveolar macrophages have also been shown to be poor APCs (135), (136), (137) whereas DC and interstitial macrophages in the lung were shown to be efficient APCs (138), (139). Alveolar macrophages have been demonstrated to be suppressive to T cells (140), (141), (142), (143). There is also data suggesting that mice in which the alveolar macrophages were depleted are protected during pulmonary tuberculosis (144). When alveolar macrophages are depleted in mice by liposome-encapsulated dichloromethylene diphosphonate (AM- mice) given intranasally, they are then completely protected from death after *M. tuberculosis*. Bacterial burdens in the lungs of these mice were significantly reduced. Granuloma formation were not observed, but increased numbers of activated T cells, as well as increases in pulmonary polymorphonuclear cells (144) infection were seen and are critical to control this infection. In

general, pulmonary macrophages do not provide assistance for T cell mitogen responses (140). In a study where DC and alveolar macrophages were injected intratracheally into rats, the DC could migrate to the lymph nodes whereas the alveolar macrophages appeared to be unable to pass through the epithelium (145). Holt et al. have shown that pulmonary DC traffic from the lung to the draining lymph nodes to present inhaled antigens to T cells after bacterial infection (140). In response to *M. tuberculosis* infection DCs shift to an antigen presenting phenotype (22), (23) and can stimulate T cells from the spleens and lungs of infected mice *in vitro* (23), (97) and in the lung of infected mice (14). *M. tuberculosis*-infected DC have been observed *in-vivo* in infected mice (146).

DC also contribute to the elimination of *M. tuberculosis* by the secretion of cytokines and creating an optimal environment for immune activation. DC produce IL-12 in response to *M. tuberculosis* infection and IL-12 production is critical to drive a TH-1 response necessary to fight infection. IL-12 production is responsible for activating T cells to produce IFN-γ. IFN-γ production activates both macrophages and DC to produce RNI that will prevent the growth of *M. tuberculosis*. Murine and human DC secrete TNF-α, IL-6, IL-1α, and IL-1β which contribute to the inflammatory response and cell migration (14), (23), (22), (147), (148). DC express CD40L and studies have shown the CD40 stimulated *BCG*-infected mice express high level of the chemokines MIP-α/β which are potent chemotactant signals for DC and neutrophils in mice (149). CD40 cross-linking on DCs has also been shown to lead to the secretion of high level of IL-12 (150), (151). DC express Toll Like Receptors (TLR) which when stimulated with mycobacterial antigens can upregulate NF-κB regulated cytokines, such as IL-1 and TNF-α. TLRs on $CD8\alpha^+$ DC have been shown to induce IL-12 production in response to *E. coli* (84). DC use TLR to discriminate different microbes and stimulate either a TH1 or TH2 response (84).

DC can present non-protein antigens to T cells via non-classical MHC class I molecules, CD1. Thus far CD1 presentation has not been shown to have an affect on conferring protection in an *M. tuberculosis* infection, even though there is clear evidence that LAM is presented by CD1b and other mycobacterial lipids are presented by CD1a and CD1d (130). However, mice do not have CD1 Group 1 antigens, so the importance of T cells restricted by CD1a, b, c is difficult to test. DC infected with *BCG* and *M. tuberculosis* induced modest protection in mice after challenge, which confirms the importance of DC in initiating a protective immune response (14). DC may also play a role in downmodulating the immune response, which in a *M. tuberculosis* infection, can cause considerable damage. DC may participate in this downmodulation by B7 engaging CTLA-4 on activated T cells which inhibits the T cells proliferation. APCs express B7-H1 that can engage inducible co-stimulatory receptors on T cells and induce IL-10 (reviewed in (152)). It is believed that tolerance may be induced by immature DC phagocytosing apoptotic cells and then being unable to stimulate T cells (153).

DC may also function as effector cells . DC were shown to produce NO (23), (55), (53), (54). NO produced by DC may play a role in autoimmune disease such as Multiple Sclerosis (MS). In the mouse model of this disease, experimental allergic encephalomyelitis (EAE) the NO produced by the DCs caused apoptosis of the autoreactive T cells (53). NO production may not always be advantageous for the host. In one study with *M. avium* it was suggested that the NO produced from macrophages did not kill *M. avium* and downregulated a TH1 response which helped the bacteria subvert the immune response (154). Overall, DC may benefit the host by exhibiting anti-microbial mechanisms that protect them from being overwhelmed and killed during an infection. In addition these mechanism may enhance their APC function since the DC

may retain live bacteria and retain the ability to stimulate T cells in a pathogen-specific manner (155).

Hypothesis and Specific Aims

My hypothesis is that DC are crucial for the initiation of a protective immune response in *M. tuberculosis*. We hypothesize that they are infected in the lungs, migrate from the lungs to the draining lymph nodes to prime naive T cells, which will return to the lung to establish protective immunity. DC at the site of infection can also contribute to the immune response by secreting inflammatory cytokines. We also hypothesize that DC will have effector functions that enable them to participate in control of *M. tuberculosis infection*.

The basis for the initiation of the immune response which leads to protection in the majority of immunocompetent people infected with *M. tuberculosis* is not well understood. Although 1.8 million people are believed to be infected, only approximately 10% of immunocompetent people will present with active tuberculosis. Therefore, in immunocompetent people a successful protective immune response is usually mounted. A cell-mediated immune (CMI) response is necessary for adequate protection. However, alveolar macrophages have not been shown to generate a strong CMI; in fact they may be suppressive for T cells. DC are potent antigen presenting cells (APC) and play critical roles in many infections and have been shown to play a role in eliciting T cell responses to airway pathogens. DC can secrete cytokines which favor a Type 1 T cell response necessary for the control of *M. tuberculosis*. We investigated these and other roles DC play in *M. tuberculosis* infection and compared these roles to that of the macrophage, which is the known host cell for *M. tuberculosis*. We also examined the intracellular environment of DC to try to identify mechanisms which would be effective against *M. tuberculosis.* These mechanisms may help to control the bacterial burden and also aid in the APC ability of the DC. Our goal was to identify the unique role that DC play in this infection.

The specific aims for this project are detailed below:

1. Examine whether murine DC are infected and mature in response to *M. tuberculosis* infection:

The infection of murine bone-marrow derived DC was examined and compared to macrophages. Various markers of maturation in the DC, such as cell surface expression, cytokine production, and phagocytic ability were examined. The antigen presenting ability of DC and macrophages cells was compared by examining the proliferation of T cells. These data provide us with information about the ability of these cells to initiate a cell mediated immune response.

2. Examine the anti-mycobacterial mechanism in DC:

We monitored the intracellular proliferation and growth of *M. tuberculosis* in untreated and IFN-γ + LPS-activated DC and macrophages. We investigated the production of RNI and the effect of these RNI on the intracellular growth of *M. tuberculosis*. These studies indicated that DC have anti-mycobacteriostatic effects which are NOS2 dependent.

3. Investigate the mechanism underlying the differences in bacterial killing between activated DC and macrophages.

M. tuberculosis grew equally well within non-activated DCs and macrophage. Activation of DCs and macrophages with IFN-γ and LPS inhibited the growth of the intracellular bacteria in an NOS2-dependent fashion. However, while this activation enabled macrophages to kill the intracellular bacteria, the *M. tuberculosis* bacilli within activated DCs were not killed. We examined the production other anti-microbial compounds, such as ROI, peroxynitrite, pH levels, intracellular compartmentalization of *M. tuberculosis*

within activated DC and macrophages to determine whether these contributed to the differences seen in killing.

CHAPTER 1

Interaction of Dendritic Cells with *Mycobacterium tuberculosis*

This chapter has been modified from:

Bodnar, K. A., N. V. Serbina, and J. L. Flynn. Fate of *Mycobacterium tuberculosis* within Murine Dendritic Cells. Infection and Immunity. **69**. 2:800-809.

Introduction

It has been established that T cells provide protection against *M. tuberculosis* [reviewed in (156)] and there are studies supporting roles for both $CD4^+$ and $CD8^+$ T cells (15, 115, 117, 125, 126, 157). How precisely cell-mediated immunity is generated in a *Mycobacterium tuberculosis* infection is not known. The interaction between DC and *Mycobacterium tuberculosis* may provide insight into how the protective immune response is generated. Macrophages, as well as DC, are monocyte derived cells which are phagocytic and can present antigens. *M. tuberculosis* resides and multiplies within macrophages, which can be activated by IFN-γ and other signals to control the infection.

The interactions between DCs and pathogens are of prime importance in establishing an appropriate immune response. It has been demonstrated that DCs internalize various microbes (22, 158-165). We reported previously that human peripheral blood-derived DCs phagocytose *M. tuberculosis* and subsequently display a phenotype consistent with that of a mature DC (22). Recently it has been reported that a murine DC cell line also can be infected with *M. tuberculosis*, which results in a mature phenotype with secretion of inflammatory cytokines (14). The initiation of a protective immune response depends on the interaction of antigen presenting cells (APC) and naïve T cells, which occurs in lymphoid organs, including lymph nodes. Dendritic cells (DCs) are considered to be the most potent APC and play a crucial role in the initiation of an adaptive immune response. Following phagocytosis of an antigen (such as a bacterium), mannose and Fc receptors on DCs are downregulated, while adhesion, antigen presenting, and co-stimulatory molecules for T cells are upregulated, resulting in a mature DC (140, 166). The murine model of tuberculosis has provided considerable insight about immune responses to *M. tuberculosis*. In this study we extend the findings from our human DC studies to

the mouse system. We present data indicating that the responses of DCs and MØ to *M. tuberculosis* infection differ and show that DC can prime an immune response which is protective against *M. tuberculosis*, one that is superior to that primed from macrophages. This may have implications for the immune response against this microbe, as well as for the persistence of bacilli in the host.

Materials and Methods

Mice:

Adult female (8-10) week old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used. NOS2-/- mice on a C57BL/6 background were generated by MacMicking, et al. (64) and kindly provided by Dr. Timothy Billiar (University of Pittsburgh School of Medicine). All mice were maintained in a specific pathogen-free Biosafety Level 3 facility.

Culture and purification of DC and MØ:

DCs and MØ were generated from the bone marrow cells of C57BL/6 mice. Briefly, cells were extracted from the femur and tibia bones of mice in DMEM medium. For the MØ cultures, cells were washed twice in DMEM and 2.5 X 10^6 cells were plated in LabTek PS petri dishes (Fisher Scientific, Pittsburgh PA) in 25 ml DMEM supplemented with 10% certified FBS, 1mM sodium pyruvate, 2mM L-glutamine (Life Technologies, Grand Island, NY), and 33% supernatant from L-cells fibroblasts cultured for 5-6 days. All reagents were LPS-free and no antibiotics were used. Medium was changed on day 3. On day 5, adherent cells were washed twice with ice-cold PBS (Life Technologies, Grand Island, NY), incubated for 20 minutes on ice, and harvested using cell scrapers (Becton Dickinson Labware, Lincoln Park, NJ). Cell

concentration was adjusted to 1.0 x 10^6 cells/ml and cells were placed in Teflon jars (1 ml) (Savillex, Minnetonka, MN) or aliquoted into a 96 well plate (200µl/well) for infection.

For DC cultures, bone marrow cells were centrifuged at 1200 rpm for 7 minutes, and red blood cells were lysed with NH_4Cl/Tris solution. T cells (CD4+ and CD8+) were removed using Low-Tox–M rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, NY) after incubation with anti-CD4 antibody (GK1.5 10µg/1 x 10^7 cells) and anti-CD8 antibody hybridoma supernatant (anti-CD8α, clone 83–15–5). Cell concentration was adjusted to 1 x 10^6 cells/ml and adherent cells were depleted by overnight culture in DC medium containing DMEM, 2 mM L-glutamine, and heat inactivated 5% mouse serum (Sigma, St. Louis, MO). The non-adherent cells were cultured at 0.25 x 10^6 cells/ml in 24-well plates (Costar, Cambridge, MA) in DC medium containing 1000 Units/ml of rm-GM-CSF and rmIL-4 (Schering-Plough, Kenilworth, NH and kindly provided by Dr. Walter Storkus). At day 5 cells, non-adherent cells, were harvested, adjusted to 1.0 x 10^6 cells/ml in DC media containing rmGM-CSF (1000 U/ml) and either dispersed into a 24 well plate (1 ml/well) or aliquoted into a 96 well plate (200µl/well) for infection.

Bacteria

M. tuberculosis strain Erdman (obtained from the Trudeau Institute, Saranac Lake, NY) was passed through mice, grown once in liquid (7H9 Middlebrook, Difco) medium, and frozen in aliquots at –80°C. Aliquots were used to start cultures at a concentration of 2.5 x 10^6 /ml in 7H9 medium; bacteria were grown in 5% CO_2 at 37°C. Cultures were used at day 6 or 7 to infect cells. The bacteria were washed and resuspended in DC or MØ media, sonicated 10s in a cup-

horn sonicator, then added to the cell cultures after estimation of bacterial numbers based on previous experience. Enumeration of viable bacteria to confirm MOI was by plating for viable CFU on 7H10 Middlebrook medium and incubated for 18 days at 37 ^{o}C with 5% humidified CO_2.

Cytospins of DCs and MØ

After culture for 5 days, 1 X 10^4 DCs and MØ in 40% FBS in RPMI medium were spun at 600 RPMs onto a glass slide in a Cytospin 3 centrifuge (Pittsburgh, PA). The slides were airdried, fixed in acetone, then stained with a Diffquick staining kit (Sigma, St. Louis, MO).

Infection of DCs and MØ

After culture for 5 days, DCs (at 1 x 10^6/ml in DC media + rmGM-CSF, without IL-4) were infected in 24 or 96 well plates with *M. tuberculosis* at an estimated MOI of 3-5. After 12 hours, unincorporated bacteria were removed by pelleting the DCs at low speed (<1000 rpm) and reculturing with fresh media. In some experiments, MØ were cultured and infected in 96 well plates; monolayers were washed to remove extracellular bacteria and fresh medium was added. In experiments involving quantitative cultures of intracellular bacterial growth, stasis or killing, the MOI was reduced to 1 and extracellular bacteria were removed after 4 hours. To estimate the percentage of infected cells for each experiment, DCs and MØ were either airdried on poly-L-lysine coated slides or grown in parallel in glass culture well slides (Nalgene) and fixed in 1% paraformaldehyde at each time point. Slides were stained by the Kinyoun method for acid–fast bacteria. For phenotypic assays, DCs and MØ were cultured for an additional 48 hours.

Transmission electron microscopy

Uninfected and *M. tuberculosis*-infected DCs were cultured as described above for 72 hours post-infection. Cells (4×10^5) were gently pelleted at 2000 rpm for 2 minutes in microfuge tubes, washed twice in PBS, and then fixed in 1.5% paraformaldehyde and 1.0% glutaraldehyde in PBS. Cells were post-fixed in 1% osmium tetroxide in PBS, dehydrated through a graded series of alcohols and embedded in Epon (Energy Beam Sciences, Agawam, MA). Thin (60nm) sections were cut using a Reichert Ultracut S (Leica, Deerborn, MI), mounted on 200 mesh copper grids, counterstained with 2% aqueous uranyl acetate for 7 minutes and 1% aqueous lead nitrate for 2 minutes, and observed using a JEOL 1210 transmission electron microscope (Peabody, MA).

Flow cytometry analysis of cell surface markers

DCs and MØ were obtained and infected as described above. Approximately 2-5 x 10^5 were aliquoted into tubes and stained for surface markers using antibodies against MHC class I (PE-conjugated anti mouse $H2D^b$ clone KH95 with control Balb/c IgG2b), MHC Class II (FITC anti-mouse $I\text{-}A^b$ clone AF6-120.1 with control mouse IgG2κ), ICAM-1 (FITC anti-mouse CD54 clone 3E2 with control hamster IgG), B7.1 (FITC anti-mouse CD80 clone 16-10A1 with control hamster IgG), B7.2 (FITC anti-mouse CD86 clone GL-1 with control rat IgG2), and CD14 (PE anti-mouse clone rm C5-3 with control PE rat IgG1) in PBS containing 20% mouse serum, 0.1% BSA, and 0.1% sodium azide (FACS buffer) for 30 minutes at 4°C. All antibodies were used at 0.2 μg/10^6 cells and obtained from PharMingen (San Diego, CA). Cells were fixed in 2% paraformaldehyde for 4-15 hours and analyzed by flow cytometry (FACSCaliber) using Lysis II

(acquisition) and Cell Quest (analysis) software (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

Phagocytosis assay

Infected or control bone marrow derived DC and macrophages were harvested from culture and resuspended at 4 X 10^5/ml in DMEM medium and kept on ice. Eight microliters of FITC-latex conjugated beads of 3-micron diameter (Polysciences, Warrington, PA) were added to the cells and mixed well. The cells were incubated with the beads for 2 hr at 37^{0}C or 4^{0}C. The cells were washed five times after incubation with ice cold FACS medium then fixed for 1 hr with 1% formaldehyde before analysis by flow cytometry (FACScan; Becton Dickinson).

Cytokine production

Supernatants from control and *M. tuberculosis*-infected DCs and MØ cultures were harvested post-infection, filtered (0.2 μM filters), and stored at –80^{0}C. ELISA antibody pairs were used to detect IL-10 (JES5-SXC1 and JES5-2A5), IL-12p70 heterodimer (C15.6 and C17.15) (BIOSOURCE International, Camarillo, CA), TNF-α (MP6-XT22 and MP6-XT3) (Pharmingen, San Diego, CA) in the supernatants. Recombinant cytokines (Pharmingen, San Diego, CA and Genetics Instituite, Cambridge, MA) were used to generate standard curves. The ELISAs were performed according to Pharmingen's protocol.

RNAse protection assay (RPA)

Determination of the levels of mRNA for the genes of interest at various time intervals after the infection was performed using a multiprobe RNAse assay system (Pharmingen, San Diego, CA). Total RNA, extracted from DC and MØ, cultured and uninfected or infected with

M. tuberculosis as detailed before, using Trizol reagent (Life Technology, Grand Island, NY). The extracted RNA was subjected to RPA according to manufacturer's instructions. Protected [^{32}P]UTP-labeled probes were resolved on a 6% polyacrylamide gel and analyzed by autoradiography. Cytokine analysis was performed using custom made probe sets specific for NOS2, IL-12p40, IL-1α, IL-1β, IFN–γ, and IL-10 (mck3) and IL-4, IL2p40, TNF-α, IFN-γ, IL-1α, and IL1β (mck2B). The expression of specific genes was quantified densitometrically (Image Quant, Molecular Dynamics, Sunnyvale, CA) relative to the abundance of housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or L32.

T cell proliferation and cytokine production assays

DCs and MØ uninfected or infected (MOI 4) as described before for 24 hours then plated in triplicate in 96-well U-bottom plates (Corning Incorporated, Corning, NY) at various concentrations to achieve 1:20–1:100 APC:T cell ratio in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% certified FBS, 1mM sodium pyruvate, 2mM L-glutamine, 25mM HEPES (Life Technologies, Grand Island, NY) and 50 μM 2-ME (Sigma, St. Louis, MO). As a source of sensitized T cells, spleens were obtained from C57BL/6 mice infected for 4-5 weeks and single cell suspensions were obtained by crushing the spleens in cell strainers (Becton Dickinson Labware, Lincoln Park, NJ). Red blood cells were lysed with NH_4Cl/Tris solution, and cells were washed twice. MØ were depleted by adherence on plastic Petri dishes for 2 h at 37°C. In some experiments, B cells were depleted by adherence on anti-IgG/anti-IgM Ab coated plates (Zymed Laboratories, Inc., San Francisco, CA). Lymphocyte-enriched splenocytes were added at 2-4 x 10^5 cells/well and cultured with different APC for 3 days. Proliferation of T cells in medium alone served as a baseline. As a positive control, cells

were stimulated with ConA (Boehringer Mannheim Corporation, Indianapolis, IN) at 5 μg/ml. Cells were pulsed for the final 12-18 h of culture with 1 μCi/well [^{3}H] thymidine (Amersham Life Sciences, Inc., Arlington Heights, IL) and the incorporation of radioactivity was measured by counting cell lysates on filters in scintillation counter. The stimulation index was determined by (cpm T cell + infected APC/cpm T cells + uninfected APC). Culture supernatants were harvested after 3 days of culture and IFN-γ production was measured by sandwich ELISA using antibodies R4-A62 and XMG1.2 (biotinylated) (Pharmingen, San Diego, CA), according to the manufacturer's protocol. Recombinant murine IFN-γ used to generate a standard curve was a gift from Genentech (San Francisco, CA).

Statistics

For statistical analysis of samples, paired and unpaired student t tests were used (Instat, v. 2.03, GraphPad Software, San Diego, CA and StatView, Abacus Concepts, Berkeley, CA). P values <0.05 were considered significant.

Results

Murine bone-marrow derived DCs have a different morphology from that of murine bone-marrow macrophages.

The bone-marrow generates pleuripotent hematopoietic stem cells which produce lymphocyte and monocyte lineages. These hematopoietic cells give rise to lymphocytes, such as T cells and B cells; monocytes, which include professional antigen presenting cells, such as macrophages and DCs; as well as granulocytes, natural killer cells, esonophil, and basophil. *M. tuberculosis* infects macrophages and this study examined their ability to affect another monocytic derived APC, the dendritic cell (DC). Although these two cell types are derived from the same precursors, macrophages and DCs become very discreet and distinct cell types due to the different growth factors they encounter as monocytes. The maturation of DC is characterized by many surface markers changes such as antigen presenting molecules, MHC class II, MHC class I; T cell co-stimulatory molecules, B7.1, B7.2, CD-40; and adhesion molecules such as ICAM-1, LFA-3. There is also a decrease in phagocytosis and receptors, such as FcR and mannose receptors (167). These changes are visualized by flow cytometry as described in the next section.

In addition to these changes there is also a morphological change to the DC after it transitions from a monocyte and this different than the morphology of a macrophage. DCs have a veiled appearance and cytoplasmic processes that can extend far out from cell. DCs have a high cytoplasm to nuclei ratio (168). A simple stain and light microscopy can reveal these morphological differences. We generated immature murine DC by culture of bone-marrow cells for five days in LPS-free media supplemented with 5% mouse serum, rmGM-CSF and rmIL-4. Murine macrophages were generated from the bone-marrow cells and cultured in media

supplemented with 10% fetal bovine serum, sodium pyruvate, glutamine, and supernatant of L-cells, which provide the growth factors for macrophages. These cells were harvested and spun onto a glass spin by a cytospin procedure then stained and viewed with a light microscope (Fig. 1). The cytospins show the unique appearance of the DC with the long processes (Fig 1A), whereas the macrophage cytoplasm is elongated and stretched it does not have any processes (Fig 1B). We can isolate both macrophages and DC from the murine bone-marrow and use these in our study to compare what *M. tuberculosis* does to these cells and in the next chapter we show what happens to the bacteria within these untreated and activated cells.

Infection of murine DCs with *M. tuberculosis.*

Immature DCs are highly phagocytic and readily take up various microbes, whereas mature DCs lose their phagocytic ability (168, 169). The DCs we generate in autologous serum with LPS-free reagents and medium have a very immature phenotype as judged by cell surface molecule expression low expression of MHC class II, B7.1, B7.2, and moderate expression of ICAM (Figure 1), morphology, and a high phagocytic ability, as determined by the uptake of FITC-labeled beads (Figure 2). Therefore, these DCs should readily take up bacteria. MØ were also derived from bone-marrow precursors and were shown to also have a high phagocytic ability (Figure 2) and are known to be readily infected with *M. tuberculosis* (170). The DCs and MØ were infected with *M. tuberculosis* at an estimated multiple of infection (MOI) of 3-5 for 12 hours. The use of a higher MOI (5-10) resulted in a higher percentage of infected cells or more bacteria per cell, but was associated with a loss of cell viability. To confirm the mycobacteria were internalized, infected DCs were examined using transmission electron microscopy (Figure

2). *M. tuberculosis* bacilli were observed within vacuoles of the DCs and multiple bacilli were often present within the cell. Bacteria free in the cytoplasm were not observed.

Murine DC can be infected with *M. tuberculosis* and was comparable to the infection of MØ.

Infection of DC resulted in 50-70% of the cells infected, as judged by staining for acid fast bacilli and this is comparable to infection of macrophages (Figure 3). Human-peripheral blood derived DC (MDDC) were compared to human-peripheral blood derived macrophages (MDM) and the MDDC were suggested to be more active in the phagocytosis of *M. tuberculosis* which resulted in a slightly higher infection and slightly more bacilli per cell then macrophages (147). We observe very similar infection *in vitro* with the murine-derived cells. It was reported that human derived DCs infected with *M. tuberculosis* at an MOI of 2.5 results in 63% of the cells infected, which is compared to the murine DCs. As well higher MOI resulted in 93% of the DC infected but caused a large amount of cell death (148). Lung derived DC have been show by acid fast staining to be infected *in vitro* with *M. tuberculosis* (146). However, these were not directly compared to alveolar macrophages. This data show that there maybe another reservoir for *M. tuberculosis* other than the macrophage.

Murine DCs and MØ respond differently to *M. tuberculosis* infection.

We previously reported that *M. tuberculosis* infection of human PBMC-derived DCs resulted in phenotypic maturation of the cells. To confirm a similar effect on murine DCs, we examined cell surface marker expression following *M. tuberculosis* infection of murine bone-marrow derived DCs and MØ by flow cytometry. The DCs generated from bone marrow were cultured in mouse serum and have a very immature phenotype. DCs infected with *M.*

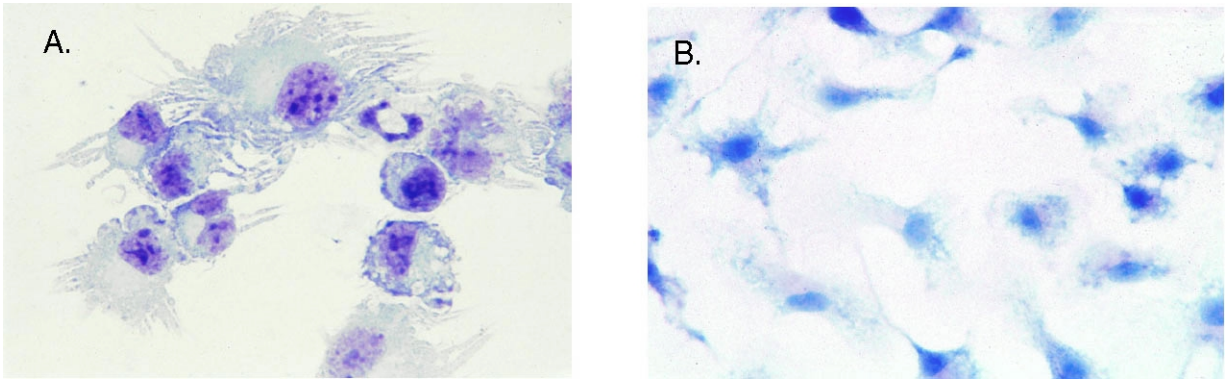

Figure 1. Morphology of bone-marrow derived DC compared to macrophages.

Cytospins of bone-marrow derived (**A**) DC and (**B**) macrophages cultured as described in Material and Methods were stained with Diffquick (Sigma, St. Louis, MO).

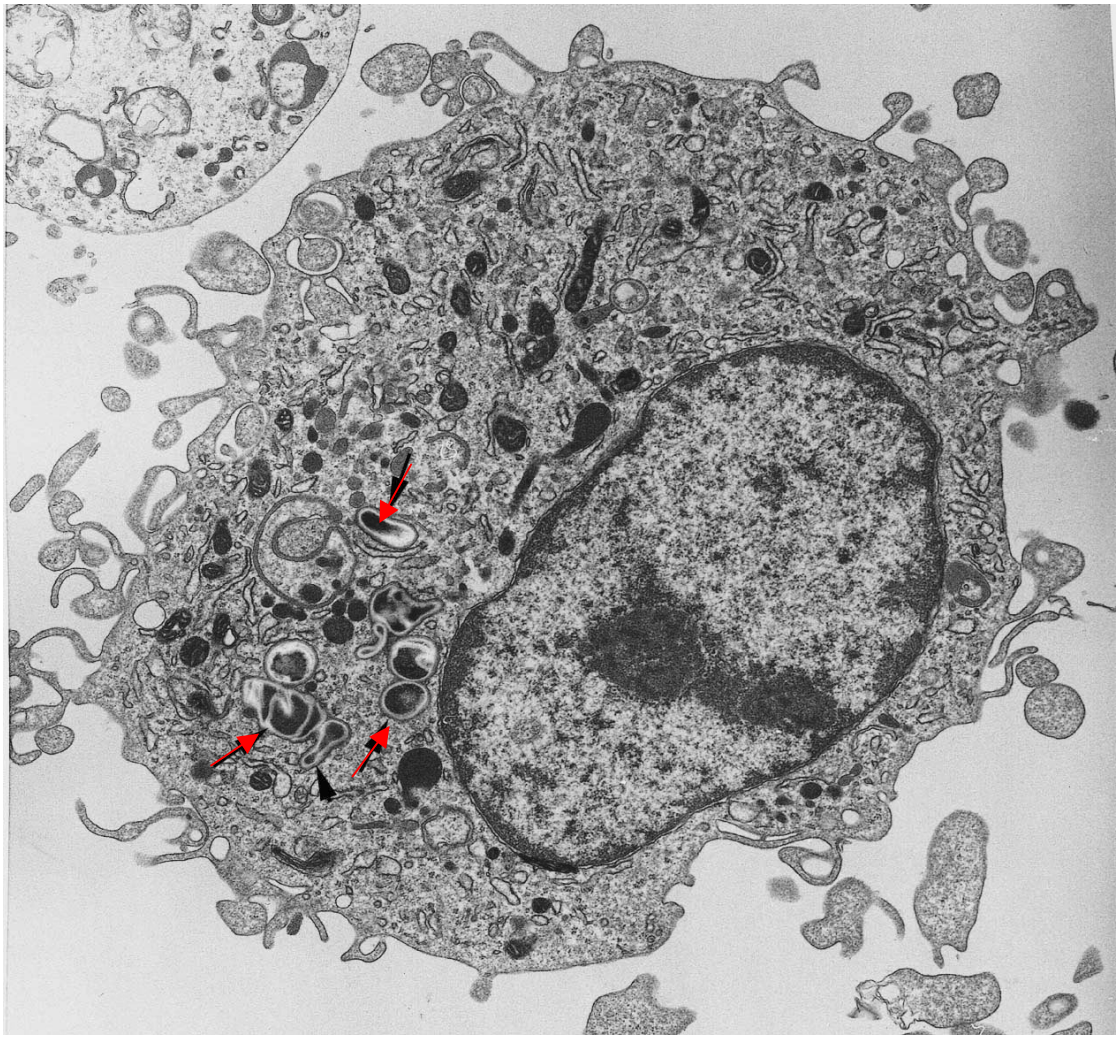

Figure 2. Immature murine DCs internalize live *M. tuberculosis.*

DCs infected with *M. tuberculosis* were fixed, embedded, sectioned, and examined by transmission electron microscopy (EM). Arrows indicated *M. tuberculosis.* The micrograph shown is representative of 25 individual cells observed.

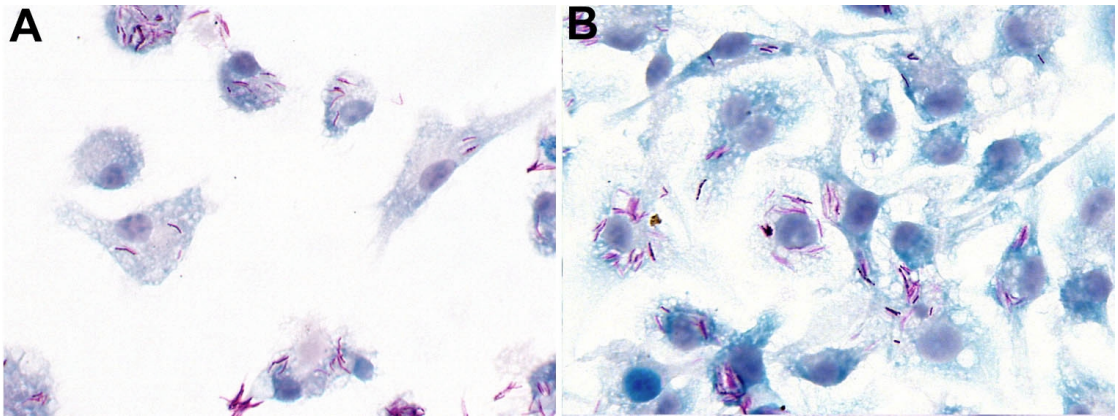

Figure 3. Murine DC can be infected with *M. tuberculosis* and was comparable to the infection of MØ.

DCs and MØ were cultured with live *M. tuberculosis* for 12 hours. DC and MØ were allowed to adhere to glass culture well slides, fixed in paraformaldehyde, and stained for acid-fast bacilli by the Kinyoun (cold) method (mycobacterium stain red). **A.** DC infected with *M. tuberculosis* and **B.** MØ infected with *M. tuberculosis*.

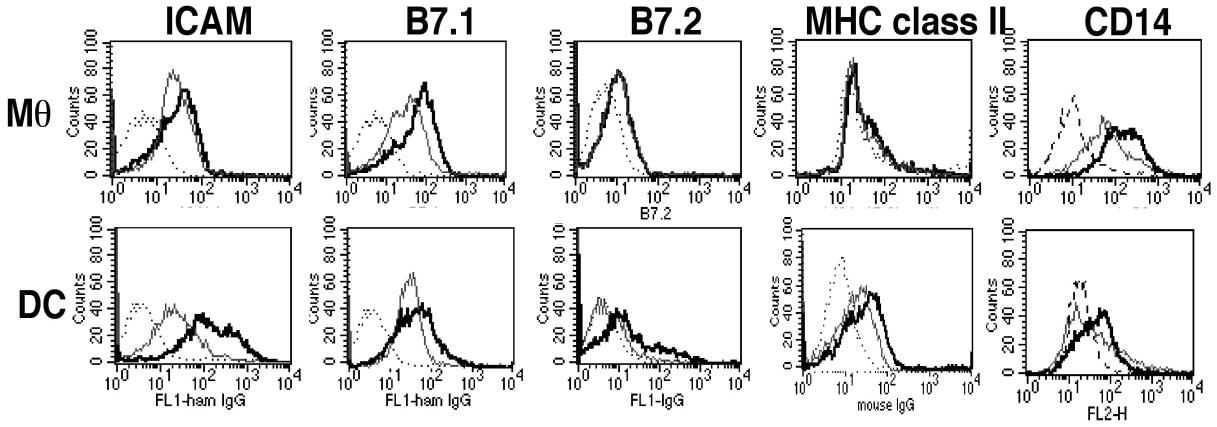

Figure 4. Cell surface phenotype of DCs infected with *M. tuberculosis*.

Purified bone marrow-derived DC and MØ were infected with *M. tuberculosis* or uninfected and harvested 48 hours post-infection. The cells were stained for IA^{b} - FITC conjugated (MHC class II**)** with mouse IgG2a, CD54-FITC (ICAM) and CD80-FITC (B7.1) with hamster IgG, CD86-FITC (B7.2) with rat IgG2a, and CD-14-PE with rat IgG antibodies, fixed, and analyzed by FACS analysis. Top row, MØ; bottom row, DC; dotted line, IgG control; thin gray line, uninfected cells; thick black line, *M. tuberculosis*-infected cells. A representative experiment of 10 independent experiments is shown.

tuberculosis showed a consistent upregulation in expression of cell surface molecules ICAM, B7.2, and B7.1, as compared to uninfected cells (Figure 4, Table 1), suggesting a shift to a more mature phenotype. Although the MØ express MHC Class I and II, B7.1, B7.2, and ICAM-1, these were not generally upregulated following infection with *M. tuberculosis*. The MØ express high levels of CD14, a receptor for LPS, which is highly expressed on macrophages. Whereas the DCs only express a small amount of CD14, which appears to be slightly upregulated upon infection and this has been described elsewhere (Figure 4, Table1) (171). The morphology of both population of cells is changed post-infection (Fig. 5) although the surface marker expression of MØ does not change much.

Immature DCs are quite phagocytic, but this is reduced upon maturation (172, 173). We compared the effect of *M. tuberculosis* infection on the phagocytic potential of murine DC and macrophages using FITC-labeled 3 micron labeled latex beads (Figure 6). Upon infection, DCs had reduced phagocytic ability at 37°C compared to uninfected DC (lower MFI and percent gated). In contrast, no change in phagocytic potential of macrophages was observed following infection (Figure 6). These data suggest that *M. tuberculosis* infection results in maturation of DCs, confirming the results obtained with human DCs (22) and to that of other infections (174).

Inflammatory cytokines induced by *M. tuberculosis* infection of DCs.

Macrophages infected with *M. tuberculosis* secrete inflammatory cytokines, which can influence cytokine production by T cells (175). The production of cytokines by DCs during priming of T cells in the lymph node is likely to affect the initiation of an immune response against an infection. IL-10, IL-12, and TNF-α secretion by *M. tuberculosis*-infected DC and MØ culture were measured. IL-12 was detectable by 12 hrs in *M. tuberculosis*-infected DCs and as

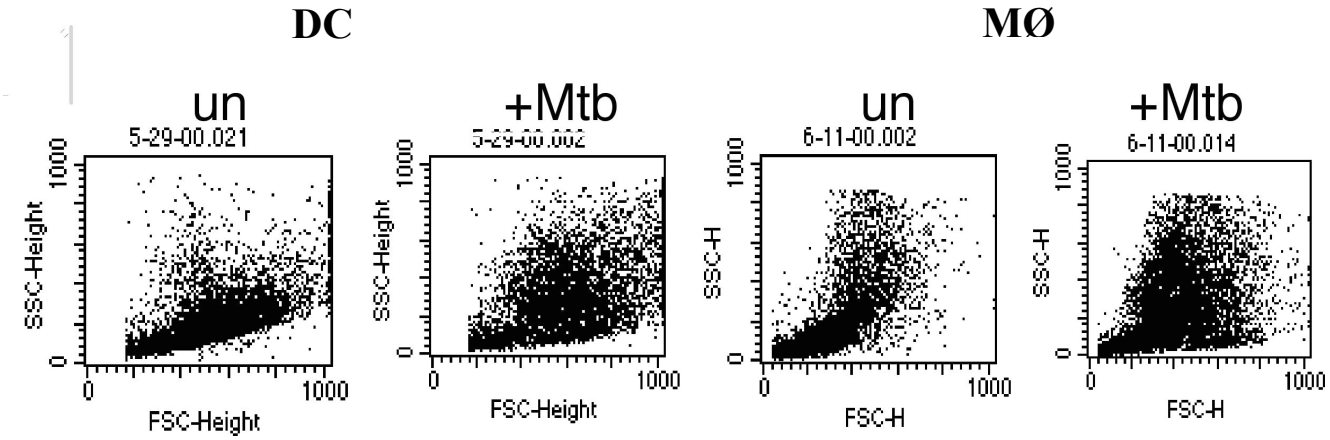

Figure 5. Cell size and density of DCs and macrophages infected with *M. tuberculosis*.

Purified bone marrow-derived DC and MØ were infected with *M. tuberculosis* or uninfected and harvested 48 hours post-infection. The cells were stained, fixed, and analyzed by FACS analysis. The forward versus side scatter plots show that both the DC and undergo morphologic changes after infection.

	MACROPHAGE					DENDRITIC CELLS				
	uninfected		+M.tb			uninfected		+M.tb		
	MFI*	%+	MFI	%+	fold increase ^	MFI	%+	MFI	%+	fold increase ^
MHC molecules										
CD14-PE	209.69	68.0	272.80	93.0	1.3	129.3	28.0	138.9	38.0	1.1
IA^6-FITC (class II)	110.0	12.6	126.0	14.	1.1	49.0	18.0	210.2	44.0	4.0
hamster IgG-PE			10.52	2.5				20.6	2.3	
murine IgG-FITC			11.5	1.8				8.96	1.4	
Co-stimulatory molecules										
CD-80 (B7.1)	84	55.0	101.0	77.	1.2	67.0	58.0	113.4	68.0	1.7
CD-86 (B7.2)	103.1	16.5	107.9	16.0	0.9	50.0	4.19	118.0	27.8	2.4
hamster IgG-FITC			6.09	1.23				4.4	2.3	
rat IgG2a-FITC			6.24	0.2				4.9	0.58	
Adhesion molecule										
CD54 (ICAM-1)	128.0	36.0	129.0	38.0	1.0	62.0	45.0	245.0	93.0	3.9
hamster IgG-FITC			6.51	0.7				4.4	1.9	

Table 1. Cell Surface Phenotypes of DCs infected with *M. tuberculosis*.

Purified bone marrow derived DCs and were infected with *M. tuberculosis* or uninfected and harvested 48 hours post-infection. The cells were stained for H-2D-PE conjugated (MHC class I), IA^{b-} FITC conjugated (MHC class II), CD54-FITC (ICAM), CD-80-FITC (B7.1), CD-86-FITC (B7.2), and CD14-PE antibodies, fixed, and analyzed by FACS analysis. Cells were gated on size (forward angle scatter) and density (side scatter) to exclude cellular debris and dead cells. The table shows a representative FACS experiment of 10 independent experiments.

* Mean Fluorescence Intensity ^ MFI compared to the MFI of uninfected cell

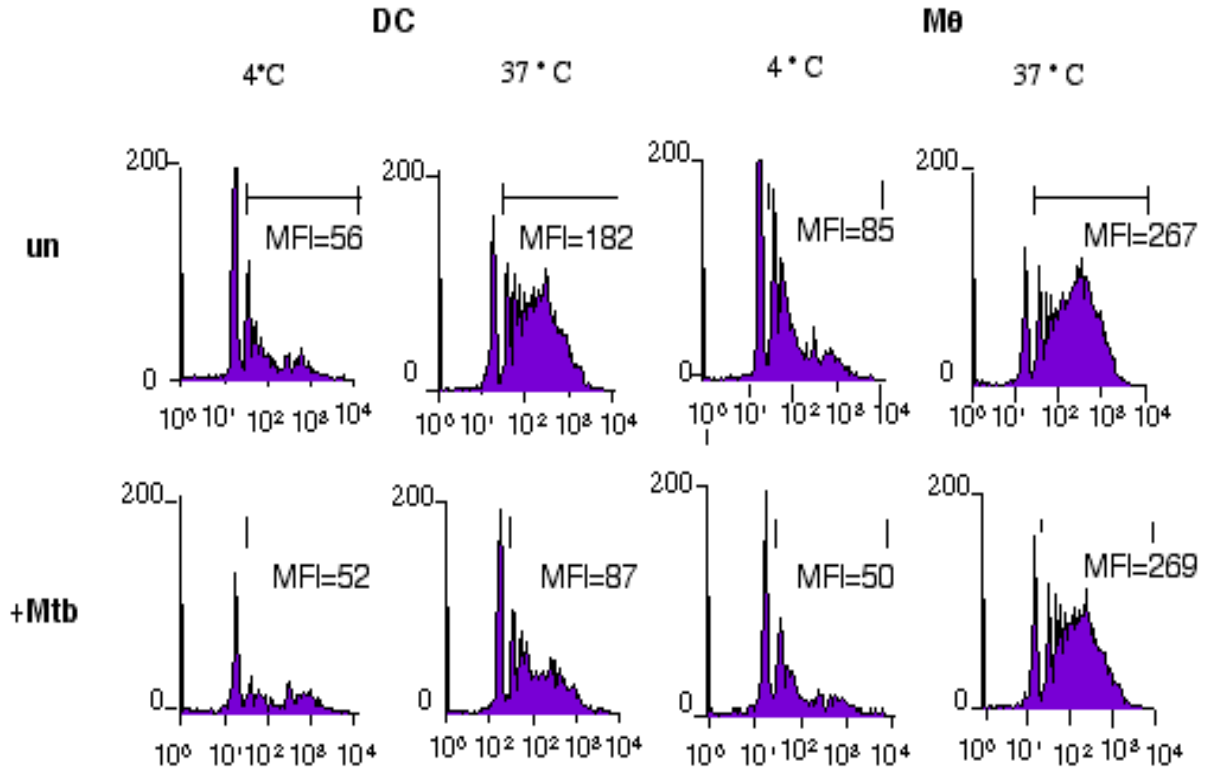

Figure 6. Phagocytic capability is decreased in *M. tuberculosis*-infected DC but not macrophages.

Purified DC and MØ were cultured without (top panel) or with *M. tuberculosis* (bottom panel). After 72 hours, cells were incubated with 3 micron FITC-labeled latex beads for 2h at 4°C (1st and 3rd row) or 37°C (2nd and 4th row) and examined by flow cytometry. Dead cells and free beads were excluded from gate and 1 X 10^4 cells were collected within the gate for each sample. Mean fluorescence intensity (MFI) is indicated in each histogram. Bar above histograph represents gate. % gated: DC uninfected:4°C= 20%, 37°C= 76%; DC +M.tb: 4°C=5%, 37°C=37%; MØ uninfected: 4°C = 33%, 37°C =93%, MØ +M.tb:4°C =14%, 37°C =83%. Experiment was repeated once.

early as 4 hrs in infected MØ, and increased over the course of infection (Figure 7A). Infected MØ produced 2-5 fold more IL-12 than infected DCs. TNF-α was produced by *M. tuberculosis*-infected DCs and MØ by 4 hrs post-infection, with decreased production at later timepoints (Figure 7B). Infected DCs consistently produced more TNF-α early compared with infected MØ but infected MØ produced more later and this did not decline as quickly. This pattern matches the pattern of RNA expression (Figure 8B) . Uninfected DC and MØ produce negligible amounts of TNF-α and IL-12 (Figure 8, time 0). We also assessed TNF-α, IL-12, and IL-10 gene expression by an RNAse Protection Assay. IL-12 gene expression was barely detectable in *M. tuberculosis* infected DCs and MØ until 24 hours; no gene expression was observed in uninfected cells. There was a noticeable increase TNF-α gene expression over uninfected cells at all timepoints (Figure 8). In addition, IL-1α and IL-1β gene expression increased with infection in both cells (not shown). IL-10 was not detected in either uninfected or *M. tuberculosis*-infected cells at any of the time points examined, by ELISA or by intracellular cytokine staining (data not shown). However, a low level of RNA was detected in *M. tuberculosis*-infected DC and MØ and this increased over time (Figure 8).

Stimulation of T cell proliferation and cytokine production by antigen presenting cells.

The differential effect of mycobacterial infection on expression of co-stimulatory molecules by DC and MØ suggested that they might differ in mycobacterial antigen presenting functions. To assess the ability of infected DCs and MØ to stimulate T cell effector functions, T cells from *M. tuberculosis*-infected mice were cultured with either DCs or MØ as APCs. Both uninfected and live *M. tuberculosis*-infected APCs were used. *M. tuberculosis*-infected DCs induced substantial proliferation of T cells from infected mice (Figure 9A). In contrast, uninfected DCs and MØ

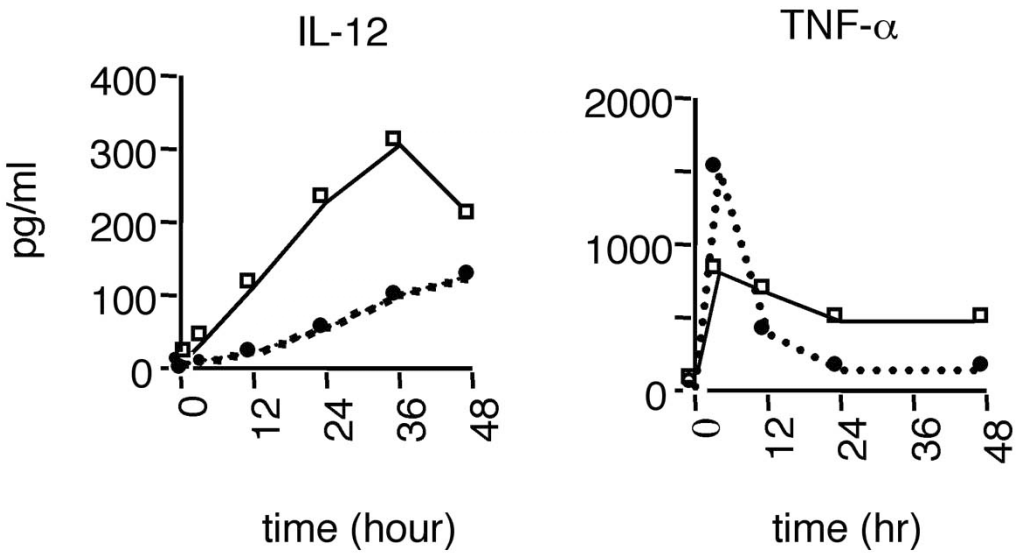

Figure 7. Cytokine secretion in response to *M. tuberculosis*-infected DCs and MØ.

Supernatants from *M. tuberculosis*-infected unactivated DCs (black circles) and MØ (white squares) were assayed in duplicate for cytokine production IL-12 (ELISA for IL-12p70) or TNF. Time 0 represents cytokine production from the cells prior to infection. Recombinant murine cytokines were used as standards in each assay. A representative experiment of 4 experiments is shown for IL-12 and 2 experiments for TNF-α.

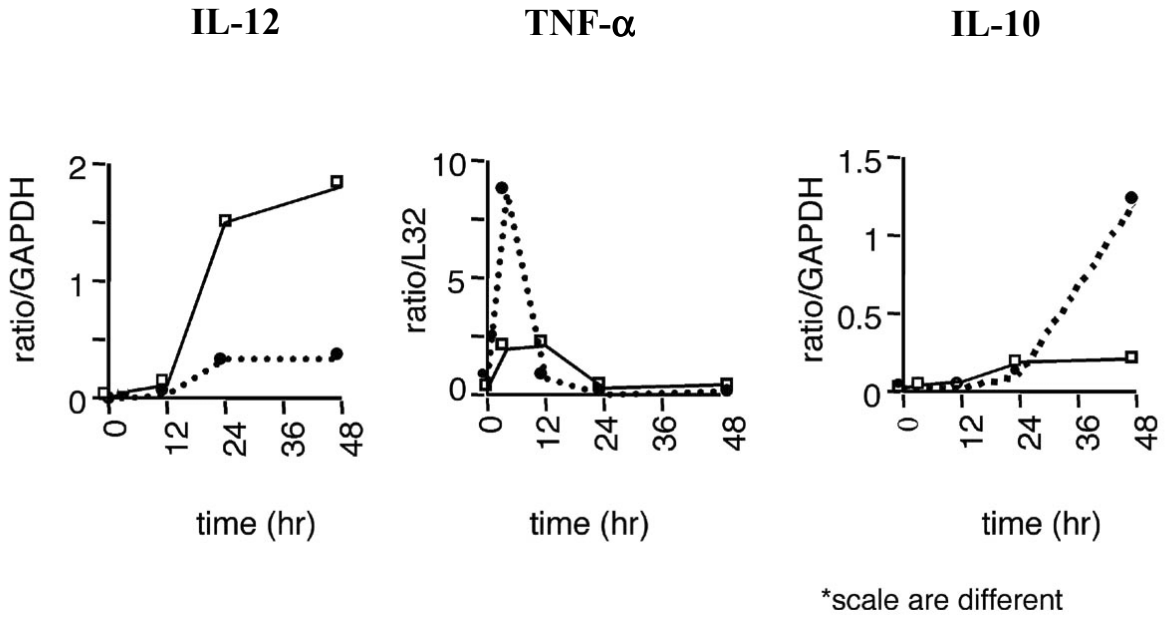

Figure 8. Cytokine gene expression in response to *M. tuberculosis*-infected DCs and MØ.

Analysis of gene expression by RNase protection assay. 6 μg of RNA from either uninfected or *M. tuberculosis*-infected DC (black circles, dotted line) and MØ (solid line, white squares) at various time points was used in the assay and specific genes of interest were standardized to GAPDH by densitometerization of the autoradiograph. A representative experiment of 2 experiments is shown.

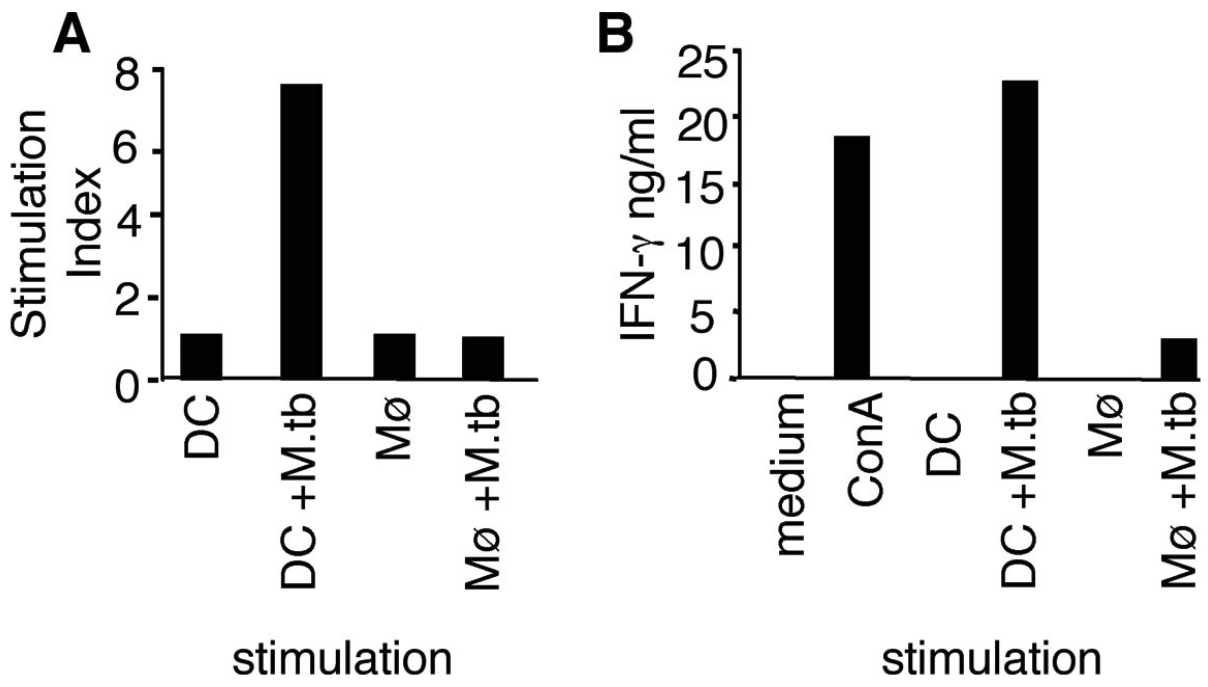

Figure 9. Stimulation of T cell proliferation and cytokine production by antigen presenting cells.

T cell enriched splenocytes from C57BL/6 mice were cultured for 3 days with bone marrow derived DC or MØ, either infected with *M. tuberculosis* (MOI 4) or left uninfected, at 1:50 ratio of APC:T cells. (**A**) Proliferation was measured by ^{3}H-thymidine incorporation and is represented as the stimulation index. Stimulation with Con A resulted in ~18,000 cpm and stimulation with infected DCs resulted in ~16,000 cpm. DCs, MØ or T cells alone incorporated fewer than 2000 cpm. (**B**) Supernatants of the cultures described above were collected at the end of the culture period and concentration of IFN-γ was measured by sandwich ELISA. A representative experiment (of 3 experiments) is shown.

and, surprisingly, even infected MØ were very poor stimulators of T cells from infected mice (Figure 9A). T cells, DCs or MØ alone did not proliferate to a significant extent (data not shown). A correlation was observed between stimulation of T cell proliferation and IFN-γ production. *M. tuberculosis*-infected DCs readily stimulated secretion of IFN-γ by T cells from infected mice, at levels comparable to that induced by Con A (Figure 9B). However, little IFN-γ secretion was observed when infected MØ, or uninfected DCs and MØ were used as APCs (Figure 9B).

Discussion:

DC readily internalized *M. tuberculosis* bacilli, and subsequently displayed phenotypic and functional changes, including upregulation of various cell surface molecules important in initiating immune responses and downregulation of phagocytic ability, as well as producing inflammatory cytokines. These DCs were superior to macrophages in stimulating proliferation and IFN-γ production of mycobacteria-specific T cells in this study (97) (14). *M. tuberculosis*-infected DC and macrophages have recently been suggested to differentially polarize naïve T cells (87). *M. tuberculosis*-infected DC biased a Type 1 (IFN-γ producing) response (TH1) response whereas macrophages did not (87). In order to develop a protective immune response to *M. tuberculosis* a Type 1 T cell response must be initiated; therefore these data suggest that the DC is a critical link to protection from disease.

Mouse bone marrow derived DC infected with *M. tuberculosis* for 48 hours demonstrated increased cell surface expression of ICAM-1, B7.1 and B7.2 molecules (both % positive cells and MFI), while decreasing their phagocytic ability and this differed from *M. tuberculosis* infection of macrophages. These data are consistent with our previous work with *M.*

tuberculosis infection of human PBMC-derived DCs, although upregulation of cell surface molecules was less profound in the murine system, and with the results reported by Tascon, et al, using the tsDC cell line (14). Although levels of B7 expression were similar between our primary DCs and the tsDC line, we also observed an increase in ICAM-1 after infection, which was not observed using the tsDC cell line (14). A consistent up or down regulation of MHC I or II was not observed in our studies. It may be that levels of MHC class I and II are susceptible to variables such as MOI, percentage of infected cells, and the number of live (or dead) bacilli per cell. We also did not observe down regulation of MHC Class II on macrophages following infection, in either this study or in our study with human DCs and MØ (22), in contrast to published reports (176-178).

Inflammatory cytokine production by DCs would be expected to influence the T cell phenotype primed in the lymph node (179). *M. tuberculosis*-infected DCs produced low to moderate amounts of IL-12, which would skew the primed T cell response toward a TH 1 (IFN-γ producing) response. The IL-12 produced directly by DC has been shown to be important in *Mycobacterium leprae* infection (149). Production of this cytokine was lower in DCs than in our *M. tuberculosis* infected MØ. IL-12 production by DCs following infection with microbial pathogens has been reported, although down regulation of constitutive IL-12 production by DC following infection with certain pathogens has also been reported (180, 181). DCs infected with *Toxoplasma gondii* have a refractory period where IL-12 production is low, but upon restimulation, IL-12 production can increase (182). In the human *in vitro* system human DCs infected with *M. tuberculosis* also produced a low but reproducible amount of IL-12 (147), (22). However, *in vitro* the human macrophages infected with *M.tuberculosis* did not produce any

detectable levels of IL-12 which makes the small amount of IL-12 produced by the DC even more critical.

TNF-α production following *M. tuberculosis* infection was initially higher in DC compared to MØ, but MØ did not decrease TNF-α production as quickly. This is similar to the pattern seen with *M. tuberculosis* infection of human DCs (22). IL-10 production was not observed in infected DC or macrophages up to 60 hours post-infection. Low levels of mRNA are detected and increased by 24 hours (Fig 4C). This down regulatory cytokine may be produced later in infection, or may be produced primarily by T cells during this infection. Recently Giacomini et. al showed that human-derived DCs produced IFN-γ inducing cytokines such, as IL-12 and IFN-α; whereas macrophages produced more pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6, as well they produced IL-10 whereas the DC did not (147). Although the cytokine profile seems to be more distinctive between the human macrophages and DCs in their system the hypothesis is that DC and macrophages play very separate functions in a *M. tuberculosis* infection and we also observe this phenomenon. Giacomini et. al. believe based on these cytokine differences that macrophages induce granulomatous inflammatory response while DCs induce an anti-mycobacterial T cell immune response. As well we have shown that DC are better at stimulating T cells and respond to *M.tuberculosis* infection in different ways, such as surface marker changes and cytokine production then macrophages.

We believe that the ability of DC excel at presenting mycobacterial antigens is important since they are capable of migrating from the site of a mycobacterial infection, most likely the lung, to the lymph nodes which drain the lung to stimulate a naïve population of T cells. DC have been shown to reside in murine lungs in the tissue, airways, and interstitium (183). Although the DC in the lung are only estimated to be less than 1% of the pulmonary

mononuclear cellular population they have been shown to be very effective APCs in the lung (183). Alveolar macrophages in particular have been shown to be suppressive in a mycobacterial infection, and resident lung macrophages are believed to be less likely to migrate from the lungs following infection. Therefore, it has been unclear as to how protective immunity is generated in a *M. tuberculosis* infection. We believe that DC are the missing link and will provide valuable information as to help protective immunity is generated.

DC have been shown to be good candidates for vaccine development against mycobacterial infections (149). Although this may not be the most practical approach for developing a vaccine against this pathogen, studies using infected DC have shown that they can generate protection from tuberculosis. IL-12-transfected DC were shown to protect against *Leishmania donovani* challenge within mice. BCG-infected-DC generated a more rapid induction of cellular immunity in mice than vaccination with BCG alone but both types of vaccination resulted in the same level of protection (184). Mice injected with DC infected with *M. tuberculosis* were shown to be protected after challenge with *M. tuberculosis* to a higher degree than the current vaccine, BCG (14). However vaccination with DC given immune stimulating cell components of *M.tuberculosis,* such as LAM, were not as effective at generating immunity as BCG in their system. These data reinforce the importance of DC in generating an immune response in infection. The data also suggest that DCs harboring live, rather than killed, bacteria can stimulate a better immune response. We next examined the intracellular viability if *M. tuberculosis* within DCs (Chapter 2).

CHAPTER 2

DC inhibit the intracellular growth of *Mycobacterium tuberculosis*

This chapter has been modified from:

Bodnar, K. A., N. V. Serbina, and J. L. Flynn. Fate of *Mycobacterium tuberculosis* within Murine Dendritic Cells. Infection and Immunity. **69**. 2:800-809.

Introduction

The fate of a microbe within a DC may affect presentation of antigen by the DC to naïve T cells. The bactericidal capabilities of DC might be expected to correspond to those of MØ, given the similarity in progenitor cell, but studies examining the fate of microorganisms within DC are rare. The production of nitric oxide by DC is dependent on activation with IFN-γ and a second signal (185). DCs encountering a pathogen in tissue, for example the lung, early in infection, are not likely to also encounter specific T cells secreting cytokines, such as IFN-γ, to activate the DC. There have been relatively few studies on the effect of DC, particularly activated DC, on intracellular bacteria. DC, due to their antigen-processing ability, have means to kill microbes, degrade them, proteolytically process their proteins for peptides to present DC are capable of killing *Chlamydia trachomatis* and *Chlamydia psittaci* by phagosome-lysosome fusion, then processing and presenting these antigens to stimulate naive $CD4^+$ cells (165).

DC are well established as potent antigen presenting cells (APC) and key to initiating a cell mediated immune response, which is particularly important for intracellular pathogens (186). Our lab and others have previously reported that DC can be infected with *M. tuberculosis in vitro*, and in response secrete inflammatory cytokines and shift to a more mature, antigen presenting phenotype. *M. tuberculosis*-infected DC are better at stimulating T cell responses than infected MØ (23) (97) (14). In this study we examined the ability of DC to control the growth of intracellular *M. tuberculosis*. DC have been demonstrated to take up various microbes, including *Salmonella* (160, 187), *E. coli* (187), *Listeria monocytogenes* (188), *Borrelia burgdorferi* (161, 189), *Bordetella bronchiseptica* (159), *BCG* (158, 190), *Leishmania* (162, 163, 191), and *Chlamydia* (164, 165, 192, 193), *Histoplasma capsulatum* (194), and *Candida albicans* (195). *Salmonella* was reported to survive and replicate within unactivated murine DC (160).

The inability of the DC to clear *B. bronchiseptica* suggested that it may contribute to the natural infection and provide a reservoir for this bacteria (159). We have reported that DC can infect human and murine DC, and we report here that unactivated DC permit the growth of *M. tuberculosis* and may also be a reservoir for the infection. *Leishmania* parasites have been shown to persist within DC in lymph nodes, suggesting that DC are impaired in their ability to kill *Leishmania* (162, 163). Recent studies indicate that certain parasitic infections, such as *Trypanosoma cruzi* and *Plasmodium falciparum,* downregulate inflammatory cytokine production and prevent maturation of the DC (181, 196). These parasites apparently manipulate DC as an immune evasion strategy.

DC, unlike MØ, are not typically considered to have effector functions against intracellular organisms. Once a microbe is ingested by a phagocyte, such as a macrophage or DC, it is usually retained in a vacuole; many intracellular pathogens can replicate and survive in the phagosome. However, it has long been known that upon activation with IFN-γ and a second signal, macrophages makes this environment quite hostile for microbes (24), (25), (26). Upon activation macrophages can produce compounds, such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), that have potent anti-microbial effects (40), (197). The vacuole containing the microbe will also proceed through the endocytic pathway to fuse with the lysosome and acidify this newly formed compartment (33). In this acidic environment, activation of hydrolytic and reactive oxygen intermediate (ROI) producing enzymes is enhanced. Compounds such as ROI and RNI can also become more potent as their half lives increase, new compounds form, or they become more reactive (56). Many viruses, bacteria, and parasites are susceptible to this environment. But, many microbes have developed means to survive. *Listeria monocytogenes* can escape the phagosome (198), (199), *Legionella pneumophila* prevents

phagosome-lysosome fusion in the host cell (200), *Coxiella burnetti* replicates only under acidic conditions (201), and *M. tuberculosis* can also prevent phagolysosome fusion (33) and (202)

M. tuberculosis is an intracellular pathogen that can often survive in the harsh environment of the macrophage. The nitric oxide produced by an activated macrophage via NOS2 dependent pathways has been shown to kill, at most, only half of the intracellular bacteria in a population of macrophages *in vitro* (61). We directly compared the growth and killing of *M. tuberculosis* in DC and macrophages to determine whether these phagocytic cells had the same capacity to kill *M .tuberculosis*. DC produce RNI, which was suggested to downregulate a strong TH 1 T cell response or kill autoreactive T cells (55), (203). Langerhans cells have been shown to produce RNI but its function was not determined (204), (205). We report here that the RNI produced by activated DC can prevent the growth of *M. tuberculosis* intracellularly. The production of RNI by macrophages activated with IFN-γ and LPS is known to be a potent means to kill intracellular organisms. RNI production by DC had not been reported to kill intracellular pathogens previously. A recent study reported that RNI did not kill *Candida albicans*, although this organism was killed within a mature DC, supposedly by lysosomal hydrolyases after phagolysosomal fusion (195).

Our data supports the idea that DC have a state beyond that of mature, in which they produce anti-microbial compounds after exposure to IFN-γ and a second signal. A recent review of nitric oxide in the immune response discusses that Langerhans cells, murine bone-marrow DC, DC cells line produce RNI in response to IFN-γ and a second signal such as LPS (206). The exception was rat thymic DC which appeared to produce RNI simply to self-antigens (203), (194). The activation of DC to produce RNI suggests that there are roles for DC beyond that of the traditional role of an antigen presenting cell. DC may be a critical link between the innate

and the CMI (207), especially in the lung (194). The ability of DC to kill microbes such as *Borrelia burgdorferi* and *Chylamdia* species by phagolysosome fusion resulted in the direct processing and presentation by MHC II, resulting in $CD4^+$ T cell stimulation. Other studies have shown that the ability of the DC to stimulate T cells was enhanced when the microbe was not killed such as *Leishmania major*(162), *Histoplamsa capsulatum* (194), *Candida albicans* (195), and *B. burgdorferi* (161). Therefore this different role for DC as an effector cell may be directly linked to its potency as an antigen presenting cell. The data we present here suggest that DC play a role in an *M. tuberculosis* infection. The differences in the ability to kill between the activated DC and MØ suggest that in this infection DC have functions which are distinct from that of MØ.

Materials & Methods

Mice

Adult female (8-10) week old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used. NOS2-/- mice on a C57BL/6 background were generated by MacMicking, et al. (64) and kindly provided by Dr. Timothy Billiar (University of Pittsburgh School of Medicine). All mice were maintained in a specific pathogen-free Biosafety Level 3 facility.

Culture and purification of DC and MØ

DCs and MØ were generated from the bone marrow cells of C57BL/6 mice. Briefly, cells were extracted from the femur and tibia bones of mice in DMEM medium. For the MØ cultures, cells were washed twice in DMEM and 2.5 X 10^6 cells were plated in LabTek PS petri dishes (Fisher Scientific, Pittsburgh PA) in 25 ml DMEM supplemented with 10% certified FBS,

1mM sodium pyruvate, 2mM L-glutamine (Life Technologies, Grand Island, NY), and 33% supernatant from L-cells fibroblasts cultured for 5-6 days. All reagents were LPS-free and no antibiotics were used. Medium was changed on day 3. On day 5, adherent cells were washed twice with ice-cold PBS (Life Technologies, Grand Island, NY), incubated for 20 minutes on ice, and harvested using cell scrapers (Becton Dickinson Labware, Lincoln Park, NJ). Cell concentration was adjusted to 1.0 x 10^6 cells/ml and cells were placed in Teflon jars (1 ml) (Savillex, Minnetonka, MN) or aliquoted into a 96 well plate (200µl/well) for infection.

For DC cultures, bone marrow cells were centrifuged at 1200 rpm for 7 minutes, and red blood cells were lysed with NH_4Cl/Tris solution. T cells (CD4+ and CD8+) were removed using Low-Tox–M rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, NY) after incubation with anti-CD4 antibody (GK1.5 10µg/1 x 10^7 cells) and anti-CD8 antibody hybridoma supernatant (anti-CD8α, clone 83−15−5). Cell concentration was adjusted to 1 x 10^6 cells/ml and adherent cells were depleted by overnight culture in DC medium containing DMEM, 2 mM L-glutamine, and heat inactivated 5% mouse serum (Sigma, St. Louis, MO). The non-adherent cells were cultured at 0.25 x 10^6 cells/ml in 24-well plates (Costar, Cambridge, MA) in DC medium containing 1000 Units/ml of rm-GM-CSF and rmIL-4 (Schering-Plough, Kenilworth, NH and kindly provided by Dr. Walter Storkus). At day 5 cells, non-adherent cells, were harvested, adjusted to 1.0 x 10^6 cells/ml in DC media containing rmGM-CSF (1000 U/ml) and either dispersed into a 24 well plate (1 ml/well) or aliquoted into a 96 well plate (200µl/well) for infection.

Bacteria

M. tuberculosis strain Erdman (obtained from the Trudeau Institute, Saranac Lake, NY) was passed through mice, grown once in liquid (7H9 Middlebrook, Difco) medium, and frozen in aliquots at –80°C. Aliquots were used to start cultures at a concentration of 2.5 x 10^6 /ml in 7H9 medium; bacteria were grown in 5% CO_2 at 37°C. Cultures were used at day 6 or 7 to infect cells. The bacteria were washed and resuspended in DC or MØ media, sonicated 10s in a cup-horn sonicator, then added to the cell cultures after estimation of bacterial numbers based on previous experience. Enumeration of viable bacteria to confirm MOI was by plating for viable CFU on 7H10 Middlebrook medium and incubated for 18 days at 37 °C with 5% humified CO_2.

Infection of DCs and MØ

After culture for 5 days, DCs (at 1 x 10^6/ml in DC media + rmGM-CSF, without IL-4) were infected in 24 or 96 well plates with *M. tuberculosis* at an estimated MOI of 3-5. After 12 hours, unincorporated bacteria were removed by pelleting the DCs at low speed (<1000 rpm) and reculturing with fresh media. In some experiments, MØ were cultured and infected in 96 well plates; monolayers were washed to remove extracellular bacteria and fresh medium was added. In experiments involving quantitative cultures of intracellular bacterial growth, stasis or killing, the MOI was reduced to 1 and extracellular bacteria were removed after 4 hours. To estimate the percentage of infected cells for each experiment, DCs and MØ were either airdried on poly-L-lysine coated slides or grown in parallel in glass culture well slides (Nalgene) and fixed in 1% paraformaldehyde at each time point. Slides were stained by the Kinyoun method for acid–fast bacteria. For phenotypic assays, DCs and MØ were cultured for an additional 48 hours.

Anti-mycobacterial activity of MØ and DC

The anti-mycobacterial activity of DCs and MØ was assessed by metabolic labeling of intracellular *M. tuberculosis* with [^{3}H]-uracil as previously described (104) (46). DCs and MØ (2 x 10^5 cells/well, duplicate or triplicate wells for each condition) were primed with IFN-γ (100 U/ml) (Genentech, Inc., San Francisco, CA) for 12-24 hours and then LPS (1μg/ml) (Sigma, St. Louis, MO) was added. Activated and resting cells were infected with *M. tuberculosis* (sonicated in a cup horn sonicator 20 seconds to reduce clumping) at an MOI of 3-5. Aminoguanidine (AG), an NOS2 enzyme inhibitor, was added to some conditions of the IFN-γ-treated and untreated cells, 4 hours prior to LPS addition and infection to inhibit NOS2 activity. 24 hours post infection cells were pulsed with 2.5 μCi of [^{3}H]-uracil, which is incorporated predominantly by the bacteria and not by MØ or DCs. Supernatants were removed 8-16 hours later, the cell pellets were lysed with 1% saponin and TCA precipitated onto GF/C glass fiber filters (Fisher Scientific, Pittsburgh, PA.) and radioactive incorporation was measured by β-scintillation counter. The percent inhibition was calculated as 100-[(cpm activated cells/cpm resting cells) X 100].

For determination of actual intracellular colony forming units (CFU), DCs and MØ cultures were prepared as above, and cell lysates at each time point were cultured on 7H10 plates (10-fold dilutions in PBS + 0.05% Tween). The number of extracellular bacteria was determined by plating the undiluted sonicated supernatant of each timepoint. The number of initial intracellular bacteria was determined at 4 hours post-infection, and reduction of input was based on that number. CFU were counted after incubation of plates at 37°C for 18 days.

Determination of nitrite accumulation

Nitrite (NO_2^-) accumulation in the supernatant of cultured cells was measured as an indicator of NO production by a Griess Assay, with a sodium nitrite standard, as previously described (208). Supernatants from 2 x 10^5 cells (100 μl) of each condition were assayed in duplicate or triplicate and absorbency was measured at 570 nm using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA).

Transmission electron microscopy

Uninfected and *M. tuberculosis*-infected DCs were cultured as described above for 72 hours post-infection. Cells (4 x 10^5) were gently pelleted at 2000 rpm for 2 minutes in microfuge tubes, washed twice in PBS, and then fixed in 1.5% paraformaldehyde and 1.0% glutaraldehyde in PBS. Cells were post-fixed in 1% osmium tetroxide in PBS, dehydrated through a graded series of alcohols and embedded in Epon (Energy Beam Sciences, Agawam, MA). Thin (60nm) sections were cut using a Reichert Ultracut S (Leica, Deerborn, MI), mounted on 200 mesh copper grids, counterstained with 2% aqueous uranyl acetate for 7 minutes and 1% aqueous lead nitrate for 2 minutes, and observed using a JEOL 1210 transmission electron microscope (Peabody, MA).

Statistics

For statistical analysis of samples, paired and unpaired student t tests were used (Instat, v. 2.03, GraphPad Software, San Diego, CA and StatView, Abacus Concepts, Berkeley, CA). P values <0.05 were considered significant.

Results

Activated DCs inhibited the proliferation of *M. tuberculosis in vitro*.

M. tuberculosis can grow within unactivated macrophages, and this is a major site for bacterial replication *in vivo*. However, murine MØ activated by IFN-γ and either TNF-α or LPS inhibit intracellular growth of *M. tuberculosis* and kill a proportion of the bacteria via NOS2-dependent reactive nitrogen intermediate (RNI) production (61). It has been reported that murine DCs can produce NO in response to LPS and/or IFN-γ (55). We examined the ability of DCs, either unactivated or activated with IFN-γ and LPS, to support or inhibit the growth of intracellular *M. tuberculosis*. Intracellular mycobacterial proliferation was assessed using ^{3}H-uracil incorporation into *M. tuberculosis*, which measures bacterial growth but does not determine whether the bacteria are killed (61, 209). *M. tuberculosis* in unactivated DCs or macrophages incorporated ^{3}H-uracil, indicating replication of intracellular bacteria (Figure 9A). DCs and MØ activated with IFN-γ and LPS inhibited the growth of intracellular *M. tuberculosis*, compared to unactivated cells, and the inhibition was comparable (76% inhibition for DCs compared to 75% inhibition for MØ). The inhibition correlated with RNI production by activated DCs or MØ (Figure 9B). Addition of an NOS2 inhibitor, aminoguanidine (AG), to both DCs and MØ prior to infection abrogated the effect on intracellular *M. tuberculosis* (Figure 9A).

Confirmation of the importance of RNI production in anti-mycobacterial activity of DCs was obtained using DCs and MØ generated from NOS2-/- mice. IFN–γ and LPS activated NOS2-/- DCs failed to inhibit *M. tuberculosis* proliferation (Figure 10), similar to activated NOS2-/- MØ (Figure 10). There was consistently a higher incorporation of ^{3}H-uracil into *M. tuberculosis* within DCs, as compared to MØ (Figure 11), although similar infection levels were

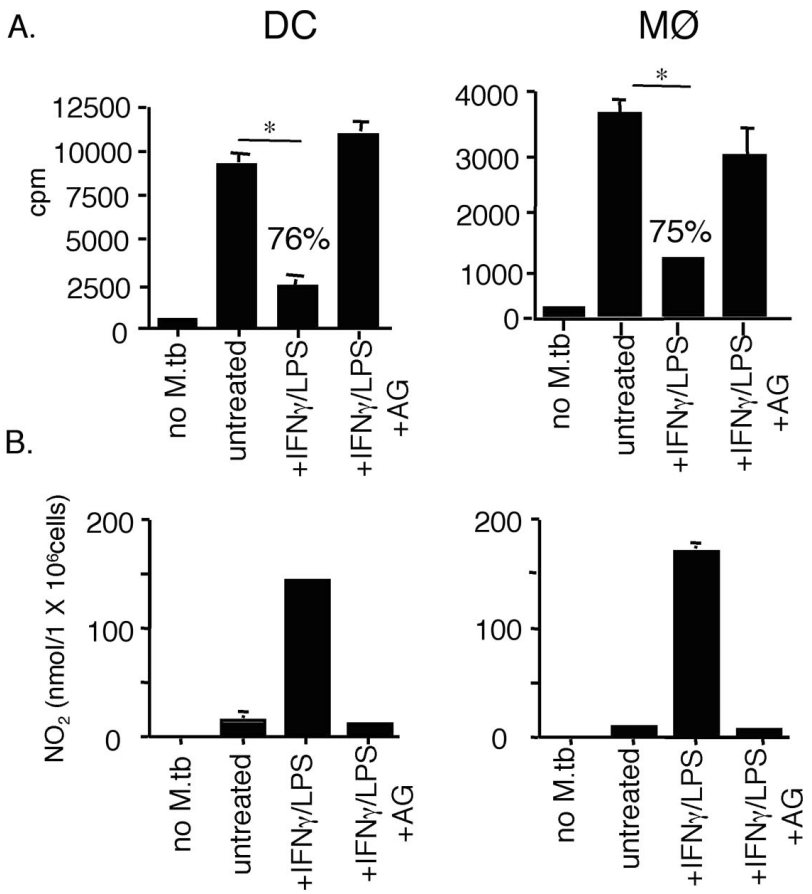

Figure 10. Activated DCs and MØ are comparable in the inhibition of *M. tuberculosis* growth via RNI production.

(**A**) Incorporation of ^{3}H-uracil into *M. tuberculosis* within DC and MØ was assessed in untreated and IFN-γ/LPS activated DC and MØ. The lysates of ^{3}H-uracil labeled uninfected, *M. tuberculosis*-infected, or IFN-γ/LPS activated *M. tuberculosis*-infected wild type DCs and MØ were TCA precipitated, collected on glass filters, and counted in a β-scintillation counter. An inhibitor of NO production, aminoguanidine (AG) was added to the cells prior to infection as indicated. (**B**) Griess assay were performed from supernatants of *M. tuberculosis*-infected DCs and MØ and read at 570 nm. The standard curve was generated using $NaNO_2$.

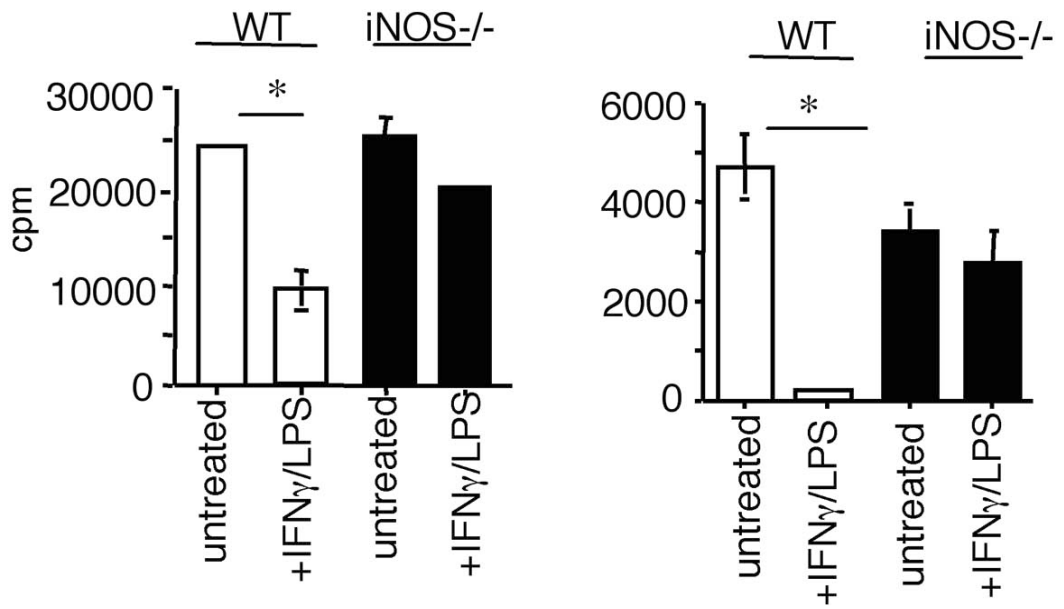

Figure 11. Killing of intracellular *M. tuberculosis* is dependent on nitric oxide in DC and macrophages.

Lysates of ^{3}H-uracil-labeled uninfected, *M. tuberculosis*-infected, or IFN-γ/LPS-activated *M. tuberculosis*-infected NOS2-/- DCs and MØ were TCA precipitated and counted using liquid scintillation. Samples were tested in triplicate and a representative experiment from 3 experiments is shown. For statistical analysis for all panels, IFN-γ/LPS or IFN-γ/LPS + AG samples were compared to untreated cells * $p<0.02$.

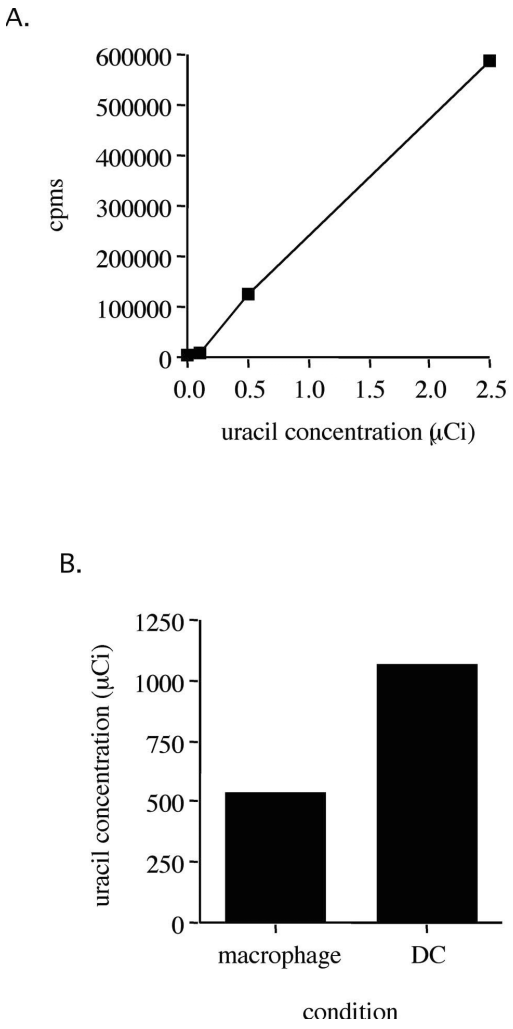

Figure 12. Tritiated-uracil incorporation and availability into mammalian and bacterial cells. (**A**.) 1 X10^6 bacilli were given various concentrations of tritiated-uracil (0.004-2.5μCi/ml). Then the cells were precipitated were TCA precipitated and counted using liquid scintillation. Cpms are reported. Experiment was repeated twice. (**B**.) DCs and macrophages obtained as detailed in Materials and Methods were given 2.5μCi/ml then lysed with 0.1% saponin, counted using liquid scintillation. Cpms are reported. Experiment was repeated four times.

obtained, as judged by AFB staining and intracellular CFU determination (see below) of each culture. To determine whether there was a differential uptake of ^{3}H-uracil by DCs as compared to macrophages, cells were pulsed with ^{3}H-uracil, washed, centrifuged, and quantitated by liquid scintillation. There was a two-fold increase in ^{3}H-uracil uptake in DC as compared to MØ (Figure 11B). DCs have a high fluid phase uptake and the additional ^{3}H-uracil inside the cell may have resulted in increased incorporation into the. Varying the concentration of ^{3}H-uracil in a cell free *M. tuberculosis* proliferation assay indicated that incorporated CPM declined linearly with the dilutions of ^{3}H-uracil (Figure 3A). These results confirmed that availability of ^{3}H-uracil affects incorporation into the bacteria. Thus, the observed differences in total CPM between *M. tuberculosis* in DCs and MØ probably reflects availability of ^{3}H-uracil, rather than any differences in bacterial growth within the cells.

Mycobacterial growth and killing within DC and MØ.

DCs encountering *M. tuberculosis* in the lung might not be activated initially, and thus the intracellular bacteria would be able to multiply, and perhaps use this cell to gain access to the lymph node. The ability of DCs to produce RNI and prevent bacterial replication suggests a potential role for DCs as an effector cell. Therefore, the anti-microbial effects of activated DCs were studied further. IFN-γ/LPS activated DCs clearly inhibited growth of intracellular *M. tuberculosis,* similar to IFN-γ/LPS activated MØ, as described above. However, the ability of DCs to actually kill the organism was unknown. To address this, intracellular CFU at various times post-infection were determined by plating lysates from an equal number of unactivated and activated infected DCs and MØ (Figure 12). Initial (4 hours post-infection) intracellular CFU were similar between DCs and MØ ($p = 0.63$). *M. tuberculosis* grew equally well within

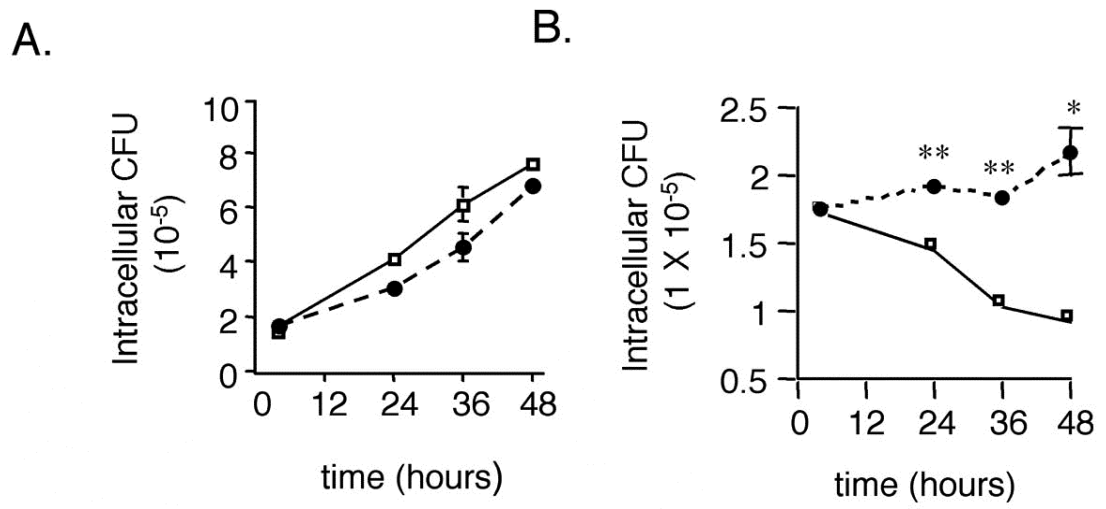

Figure 13. Dendritic cells inhibit the growth of, but do not kill intracellular *M. tuberculosis*.

Cell pellet lysates of *M. tuberculosis*-infected DCs or MØ (MOI 1), unactivated or activated with IFN-γ/LPS, were serially diluted in PBS+0.5% Tween-80 and plated on 7H10 plates which were incubated for 18 day at 37^{O}C and 5% CO_2. Three wells per condition were assessed at each time point, and the mean intracellular CFU are reported at various time points after infection. (**A**) Intracellular CFU in resting DCs (black circles) and resting MØ (open squares). (**B**) Intracellular CFU in activated DC (black bars) and activated macrophages (white bars). At each time point, <1% of total CFU was found in the supernatant. In panel A, CFU within unactivated DCs and MØ at each time point revealed no statistical differences ($p > 0.5$). In panel B, bacterial numbers within DC were not significantly reduced ($p = 0.2$) compared to intracellular CFU at 4 hours (input). Comparison of CFU within MØ at each time point to the CFU at 4 hours revealed statistically significant reductions over time (**$p<0.01$, *$p< 0.02$). Error bars show standard error. A representative experiment of 6 experiments is shown.

MØ

time (hour)	CFU (1 X 10^{-5}) [a]		fold change in CFUs [b]		NO_2^- [c] (nmol)	
	untreated	IFNγ/LPS	untreated	IFNγ/LPS	untreated	IFNγ/LPS
4	1.73 +/- 0.05	1.70 +/- 0.13			10.97	105.50
24	4.18 +/- 0.06	1.43 +/- 0.10	+2.4	- 1.1	23.80	76.80
36	6.20 +/- 0.64	1.01 +/- 0.07	+3.6	-1.6	50.50	142.70
48	7.65 +/- 0.07	0.89 +/- 0.15	+4.5	-1.9	43.5	130.5

DCS

time (hour)	CFU (1 X 10^{-5}) [a]		fold change in CFUs [b]		NO_2^- [c] (nmol)	
	untreated	IFNγ/LPS	untreated	IFNγ/LPS	untreated	IFNγ/LPS
4	1.75 +/- 0.05	1.74 +/- 0.06			0	37.5
24	3.14 +/- 0.26	1.91 +/- 0.11	+1.8	+1.1	0.14	90.9
36	4.64 +/- 0.50	1.83 +/- 0.30	+2.6	+1.1	2.31	150.6
48	6.90 +/- 0.07	2.15 +/- 0.16	+4.3	+1.2	1.74	135.3

Table 2. Activated macrophages kill *M. tuberculosis* whereas activated DCs inhibit the growth of *M . tuberculosis*.

[a] the mean of the triplicate intracellular CFU of the untreated (resting) and IFNγ/LPS (activated) cells.
[b] fold change of intracellular CFU at each timepoint compared to the intracellular CFU at 4 hours (input).
[c] nitrite in the supernatant of these samples as determined by a Griess assay.
Shown is a representative experiment of 6 performed.

These data are graphically represented in Figure 4.

unactivated DC and MØ over 60 hours (Figure 12, Table 2) confirming the ability of DCs to support the growth of intracellular *M. tuberculosis.* In activated MØ (treated with IFN-γ and LPS) there was a differential uptake of ^{3}H-uracil by DCs as compared to MØ, both cell types, the number of viable bacteria was reduced by ~50% by 48 hours post-infection, compared to 4 hours post-infection ($p = 0.02$) (Figure 12B, Table 2). The number of viable bacteria in activated DCs did not increase over time, confirming that these cells can inhibit mycobacterial replication. However, in contrast to activated MØ, the number of intracellular *M. tuberculosis* in activated DCs were not reduced over the course of the infection in six independent experiments (4 hours vs. 48 hours, $p = 0.21$), demonstrating a lack of killing of intracellular bacteria (Figure 4B, Table 1). Examination of the viability of the DC and MØ cultures over the course of infection was performed by staining parallel cultures of infected cells in chamber slides, and staining with trypan blue or for acid fast bacilli. At 48 hours, there was no difference in the viability of the DCs and MØ, although at later time points (72-90 hours) there was a marked deterioration of both cell types. For this reason, we compared intracellular killing only up to 48 hours post-infection. In addition, the culture supernatants at each time point were plated to determine the number of bacteria that had escaped the cells, perhaps due to cell death. In all cases up to 48 hours, very few bacteria (<1% of total bacterial numbers) were present in the supernatants, and there was no difference between DC and MØ cultures. Thus, the discrepancy in killing intracellular organisms between DCs and MØ cannot be attributed simply to differences in cell viability.

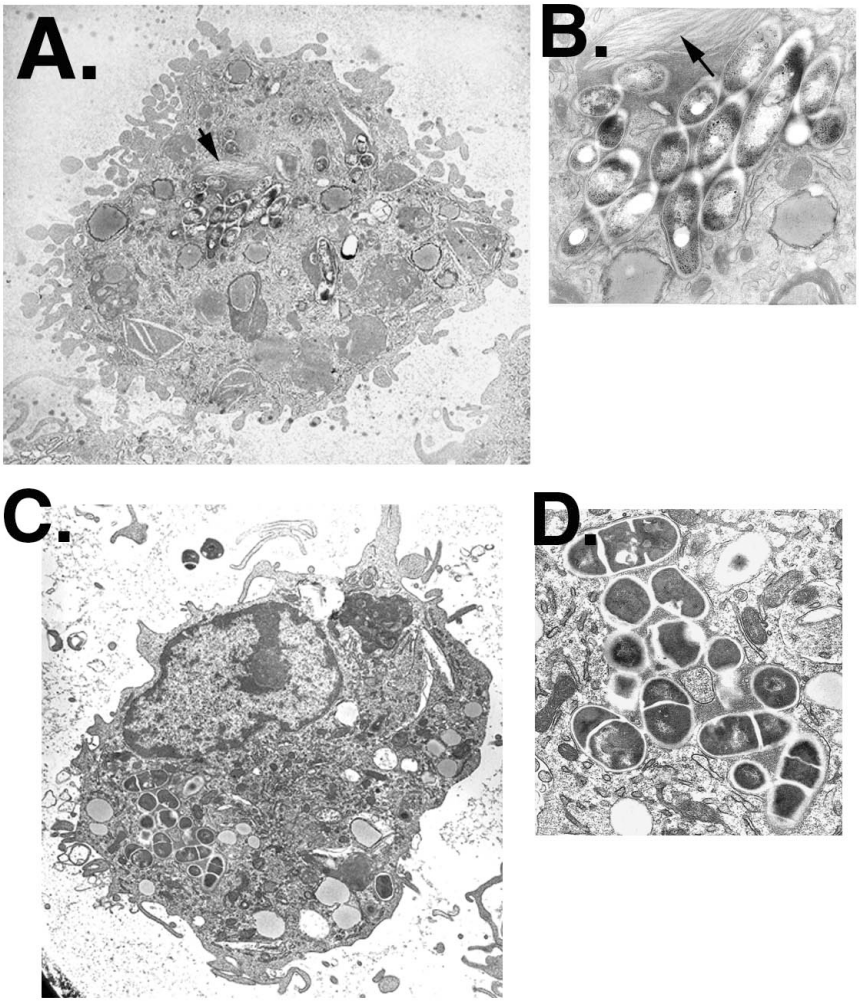

Figure 14. Activated DCs have bacteriostatic effects on intracellular *M. tuberculosis* whereas activated macrophages are bactericidal.

DCs (**A, B**) and macrophages (**C, D**) activated with IFN-γ and LPS then infected with *M. tuberculosis* were fixed, embedded, sectioned, and examined by transmission electron microscopy (EM). Arrows indicate multi-membrane.

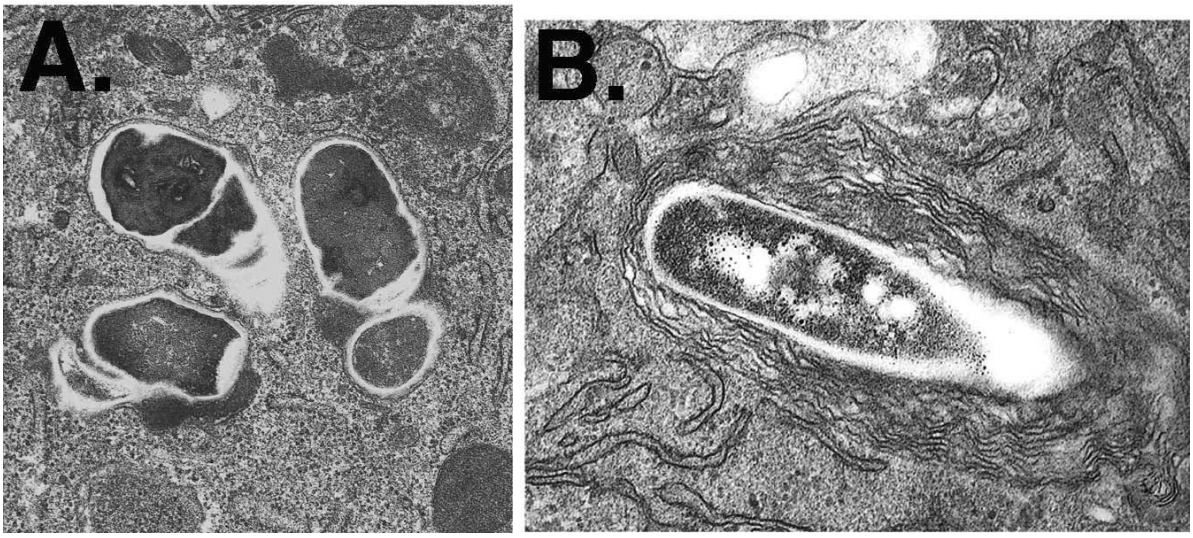

Figure 15. *M. tuberculosis* within activated macrophages is killed.

M. tuberculosis within IFN-γ and LPS activated DC (**A**) and macrophages (**B**) examined by transmission electron microscopy (EM). DC and macrophages were activated with, infected, then fixed, embedded, and sectioned. Arrows indicate multi-membrane structures that suggest bacterial killing. This figure is a representative experiment from at least 10 individual cells at several timepoints.

Electron microscopic comparison of activated DC and macrophages infected with *M. tuberculosis*.

Although activated DCs can prevent intracellular growth of *M. tuberculosis* the intracellular bacteria were not killed, as discussed above. We used electron microscopy to DC appeared viable whereas in activated MØ there were clear indications that there had been bacteria killing (Figure 13 & 14). In activated MØ we saw multi-membranous structures surrounding the bacilli and these were not observed in activated infected DC (Figure 13 & 14, arrows shows multi-membranes). The build-up of these membranes is probably due to the high composition of lipids in the mycobacteria. Structures such as this have been reported after phagocytosis of cells, where the lipid debris from the cells swirl around the membrane of the aged organelles (210). Alveolar macrophages, which are responsible for the surfactant turn-over have similar structures from the lamellar phospholipid material (210). We did not observe any other striking differences in terms of compartmentalization with activated DC compared to activated MØ. We did observed that bacilli resided singly in both resting DC and MØ, but post-activation the bacteria were seen in a communal vacuole as previously reported (32). NOS2-/- mice (and macrophages) cannot produce RNI and do not kill *M. tuberculosis*. In NOS2-/- bone-marrow derived macrophages, there was no detectable killing of the bacteria by electron microscopy (Figure 16 C,D) and there was observable killing in the WT bone-marrow derived macrophages (Figure 16 A, B).

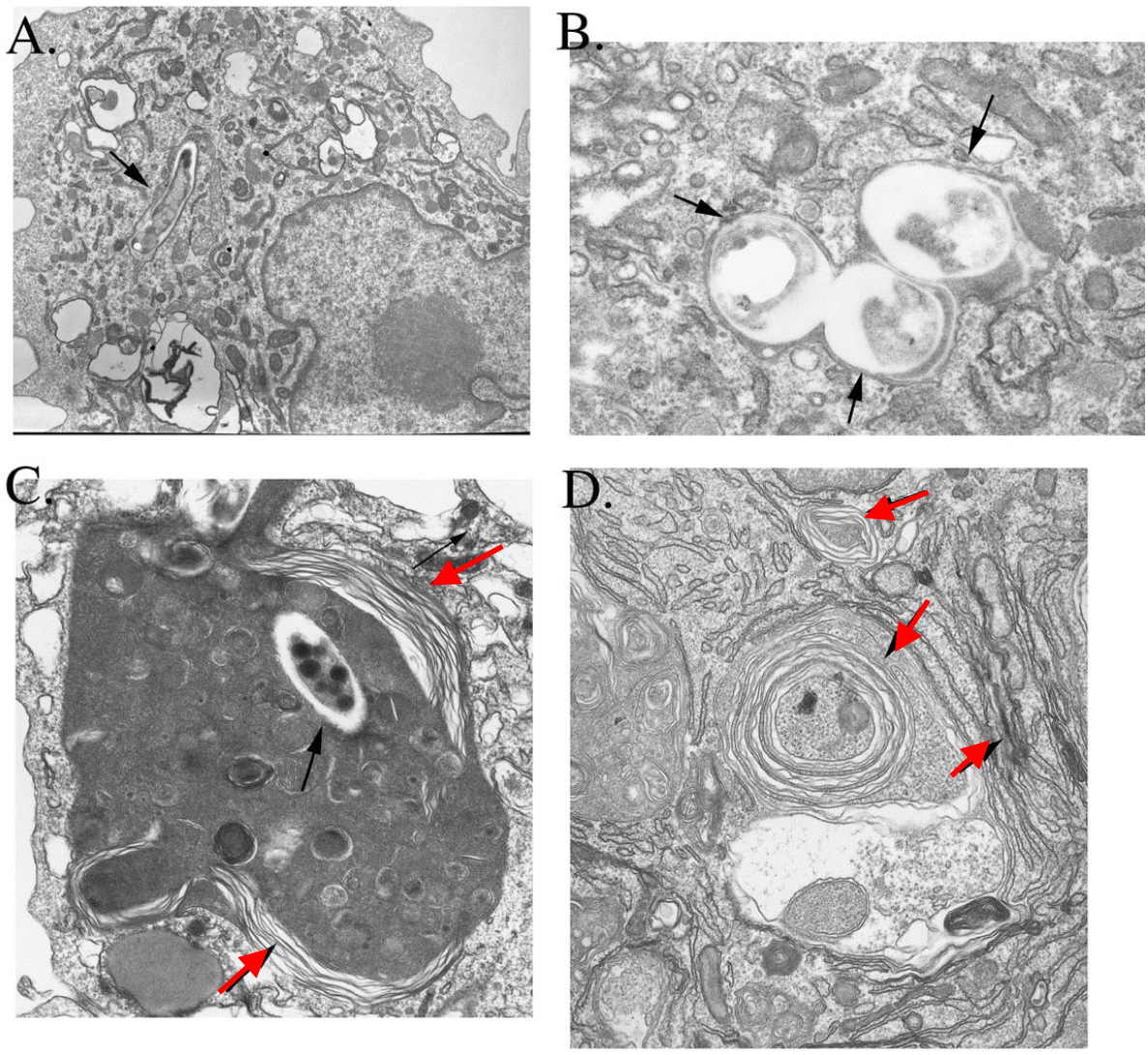

Figure 16. Activated macrophages have bactericidal effects on intracellular *M. tuberculosis* which is abrogated in macrophages from NOS2-/- mice.

Macrophages from WT mice (**A**, **B**) and NOS2-/- mice (**C**, **D**) were activated with IFN-γ and LPS then infected with *M. tuberculosis,* fixed, embedded, sectioned, and examined by transmission electron microscopy (EM). Black arrows indicate bacteria and red arrows indicate multi-membrane structures that suggest bacterial killing. This figure is a representative experiment from at least 20 individual cells at 24 hours post-infection.

Discussion

Macrophages are the known host cell for *M. tuberculosis*, however, we demonstrated previously that both human and mouse DC were easily infected with *M. tuberculosis* (22) (Chapter 1). In this chapter, we demonstrate that M. *tuberculosis* bacilli replicated equally well within unactivated DCs and MØ. Thus, maturation of the DC in response to *M. tuberculosis* infection had little effect on intracellular survival and growth of the microbe (Figure 13, Table 4). Although it has been reported that untreated murine bone-marrow-derived DC were as permissive for *M. tuberculosis* growth as bone-marrow derived macrophages others have reported that the bacteria did not grow in an untreated DC cell line (14). The disparity in results may be due to differences between primary DC and an immortalized line. Infection of human dendritic cells with a MOI of 0.1-12.5 of *M. tuberculosis* resulted in 15% to 93% infection (148). *M. tuberculosis* continued to grow in these unactivated cells for up to seven days post-infection (148). The average doubling time for *M. tuberculosis* within these cells was 26 hours. These data suggest that DC may harbor and be a reservoir for *M. tuberculosis in vivo*. As well this has been suggested for murine DC infected with *B. bronchiseptica*(159) and *Leishmania major* (163). DC may contribute to the longevity of a *M. tuberculosis* infection. A mature DC would be expected to traffic to the lymph nodes, and our data suggest that such a DC would be carrying live *M. tuberculosis* bacilli. This may be a mechanism by which *M. tuberculosis* gains access to the lymph node. Studies in the *Leishmania* murine model suggest that DC persist in the lymph node even when infected (163). Viable parasites from DC isolated from *in vivo* infection of *Leishmania major* have been isolated and used to re-infect mice and cause lesions (163). We show DC infected with *M. tuberculosis* cannot kill and eliminate the bacteria but do prevent its growth, thus allowing DC to proceed to the lymph nodes with viable bacteria (Figure 13, table 2).

It has been hypothesized that the host's ability to control an infection is a complex balance between a low number of persistent organisms and the specific effector cells in the immune system (155, 163, 211). Therefore, a continuous supply of antigen from a living bacterium may be advantageous for the priming and maintenance of an effective immune response. In experiments using *Histoplasma capsulatum* (194) and *Candida albicans* (195) there is much better stimulation of $CD4^+$ cells by DC which are infected with live microbes as opposed to the heat killed. An important consideration for vaccine development and design may be targeting DC *in vivo*. In two recent studies, mice vaccinated with BCG- or *M. tuberculosis*-infected DC were shown to generate a protective immune response and reduce the bacterial burden after challenge with *M. tuberculosis* (14, 184). Those studies suggest that targeting DC *in vivo* will be an important consideration for vaccine development and design. *M. tuberculosis*-infected DC were shown to generate a more protective immune response then BCG vaccination, however DC pulsed with immuno-stimulatory mycobacterial components did not generate more protection than BCG vaccination in mice (184). These data reinforce that live *M. tuberculosis* within DC is important for the generation of good protection.

In an environment where T cells or other cells are producing IFN-γ, DC would be expected to be activated in a manner similar to MØ. Activated MØ have been shown by various groups to inhibit the growth of intracellular *M. tuberculosis* bacilli (61, 62, 212) and, more importantly, kill at least 50% of the intracellular mycobacteria (61) via NOS2 dependent mechanisms. In our studies, activation of MØ with IFN-γ and LPS also resulted in a 50% reduction in intracellular *M. tuberculosis* by 48 hours post-infection, as determined by CFU in cell lysates (Figure 13, Table 2). Electron microscopy of *M. tuberculosis* within activated DC as compared to activated macrophages support that DC retain live viable bacteria. The bacilli in

activated macrophages are sometimes surrounded by multi-membraneous structures which are an indication of cellular breakdown by phagocytic cells. In many cases the bacilli itself appears to be degraded (Figure 14 & 15). This is not seen within activated DC and is also not readily detectable in macrophages derived from NOS2-/- mice which do not kill *M. tuberculosis* due to lack of RNI production (Figure 16). DC treated with IFN-γ and LPS were capable of restricting the growth of intracellular *M. tuberculosis*, and this was dependent on NOS2 activity (Figure 10 and 11). However, in contrast to MØ, activated DC were unable to reduce the intracellular bacterial numbers over time, despite essentially similar levels of RNI production between DC and MØ. In some experiments there were differences in RNI production at 4 hours post-infection (Table 2). Initially we speculated that more RNI produced by activated MØ early in the infection compared to activated DC could be responsible for the differential ability of the cells to kill *M. tuberculosis.* However, analysis of data from a series of experiments indicated that regardless of the RNI production by DC at 4 hours, including experiments where the output was similar to that of MØ, the DC did not reduce the number of input bacteria over time. It is possible that *M. tuberculosis* within DC avoids the killing effects of RNI by persisting within special vacuoles of DC. Other possibilities for the lack of mycobacterial killing include differences in phagosome pH, lysosomal enzymes, and reactive oxygen production in DC compared to MØ. Investigations into the mechanisms by which DC and macrophages differ are presented in chapter 3.

We hypothesize that the ability of *M. tuberculosis* to survive, although perhaps not replicate, within activated DC may be beneficial in priming a T cell response by mature DC in the lymph node. Live bacteria are capable of secreting antigens, which are believed to be important in the protective T cell response against tuberculosis. It has been suggested that *M.*

tuberculosis forms a pore within the phagosomal membrane which allows access to the cytoplasm for mycobacterial peptides; presumably priming of MHC Class I restricted CD8 T cell responses would be more efficient in DC harboring live, rather than dead, tubercle bacilli (133). An alternative hypothesis is that *M. tuberculosis* has evolved a strategy to evade killing by the DC, and uses this cell as a vehicle for disseminating from the lung to lymph nodes and other organs. As previously mentioned viable *Leishmania* parasites were isolated from the DC within the lymph nodes of infected mice. Although DC are the major APC in anti-leishmanial immunity *in vitro* (213)and *in vivo* (214), the ability to persist and to migrate to the lymph node may be a factor which prolongs this infection and lends to it's chronicity. The interactions between *M. tuberculosis* and the immune system is a constant battle, in which those traits beneficial to the generation of protection may also lend to the chronicity of the infection. DC may also have multifaceted roles as a reservoir, as an effector cell that is capable of hindering growth, and an APC which is capable of initiating a necessary cell mediated response.

CHAPTER 3

Anti-Microbial Mechanisms of Murine DC

Why is *Mycobacterium tuberculosis* not killed?

Introduction:

The interaction of dendritic cells (DC) with *Mycobacterium tuberculosi*s may play an important role in protection and one that is distinct from macrophages (MØ). In this study we examined anti-microbial mechanisms of IFN-γ/LPS-activated DC that play a role in *M. tuberculosis* infection. DC, unlike MØ are not typically considered to have effector functions against intracellular organisms. However, activation of murine bone-marrow derived DC with IFN-γ and LPS prevents the intracellular growth of *M. tuberculosis*. Unexpectedly, this activation does not appear to kill the organisms (Chapter 2). In contrast, similar activation of MØ leads to the killing of intracellular *M. tuberculosis* (Chapter 2). It is well established that the ability of activated murine MØ to kill intracellular *M. tuberculosis* is dependent on reactive nitrogen intermediate (RNI) production (61). Activated DC and MØ produce a comparable amount of RNI but there is a clear difference in the DC's ability to kill the organism. Differences in NOS2 expression at the protein and RNA level do not result in a difference in the level of nitrites produced. These data suggest that overall RNI production is not directly responsible for the differences in the ability of DC and MØ to kill *M. tuberculosis.*

The production of ROI by human derived-DC only has been reported after infection with the fungus, *Candida albicans* (195). Polymorphonuclear cells kill *C. albicans* predominately by the production of toxic oxygen metabolites, but in the human DC the production of ROI did not result in killing of this organism (195). The role of reactive oxygen intermediates (ROI) in a mycobacterial infection has been controversial. ROI are also produced by *M. tuberculosis*-infected MØ and a number of ROI including superoxide ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) can interact with nitric oxide (•NO) to form the noxious compound $ONOO^-$ (61) (57). There are

data that argue against an important role for ROI in killing or hindering the growth of *M. tuberculosis* and other data has supported a role for ROI in protection. In macrophage lines deficient in ROI production, there in no difference in the killing of *M. tuberculosis* compared to wild-type macrophages (215). *M. tuberculosis* wall component, lipoarabinomannan (LAM), is proficient at scavenging ROI generated by the respiratory burst of infected macrophages. (215). Other mycobacterial components such as sulfatides and phenolglycolipid I (PGL-1) have also been suggested to interfere with ROI-dependant anti-microbial mechanisms within macrophages (215). *M. tuberculosis* can produce both superoxide dismutase (42) and catalase (43) which could interfere with toxic oxygen radical production. On the other hand, patients with the rare genetic disorder, human chronic granulomatous disease (CGD), that renders cells unable to produce reactive oxidants (ROI) production via a defect in the NADPH oxidase complex have a higher incidence of mycobacterial infections (40). The mouse model for this disease, $p47^{phox-/-}$ or $p91^{phox-/-}$ mice, show an initial increase in bacteria but later can control the infection (216).

Peroxynitrite is a strong oxidizing compound formed by the simultaneous production of superoxide and NO (56), which we show that both occur in macrophages and DC after *M. tuberculosis* infection. Peroxynitrite production was shown to be present in blood-derived human monocytes (PBMC) and in a human monocyte derived cell line, U937 (217). Peroxynitrite can kill several intracellular pathogens, such *as Rhodococcus equi*(218), *Escherichia coli* (219), *Salmonella enterica* (68)*, Salmonella typhimurium* (220), *Mycoplasma pulmonis*(221), and *Candida albicans* (222); but many organisms are more susceptible to NO alone and not susceptible the products of ROI and NO such as *Leishmania major* (223), *Giardia lamblia* (224), *Cryptococcus neoformanis* (225)*,* and *M. tuberculosis* (49). *Leishmania major* was shown to be completely resistant to high doses of peroxynitrite (49). It has been suggested

that the addition of superoxide lowers the NO concentration and reduces peroxynitrite formation which would reduce the toxicity against organisms such as *Leishmania major* (223), *Giardia lamblia* (224), *Cryptococcus neoformanis* (225), and *M. tuberculosis* (49). Recent studies show that some bacteria, including *M. tuberculosis,* possess peptide methionine sulfoxide reductase that can protect bacteria from the oxidative damage of RNI, such as peroxynitrite. *Helicobacter pylori* protects itself from the bactericidal effects of peroxynitrite damage by carbon dioxide production (226). Pathogens may have evolved defenses mechanisms specifically for the protection from peroxynitrite. In a recent study by Yu et. al.it was demonstrated that virulent strains of *M. tuberculosis*, such as Erdman and clinical isolates, were completely resistant to peroxynitrite, yet susceptible to nitric oxide and nitrogen dioxide killing. However, non-virulent and non-pathogenic strains were highly susceptible to peroxynitrite, nitric oxide, and nitrogen dioxide killing (49). Based on these data we hypothesized that macrophages may produce more peroxynitrite, and therefore less of other, more toxic RNI, compared to DC. This is tested in the experiments detailed in this chapter.

An importance feature of the activated state of macrophages is that the endosomal compartment, which contains the pathogen (phagosome), proceeds through a pathway of maturation that ultimately leads to the fusion of the phagosome with the lysosome. This fusion creates an acidified environment which then activates many degrading enzymes and may enhance anti-microbial compounds resulting in the death of many pathogens within this phago-lysosome (28). Data has shown that *M. tuberculosis* will be killed in acidified phagosome (201). However, the bacteria has been shown to be capable of preventing this acidification, the activation of cells is thought to overcome this inhibition in mycobacterial phagosomes (227), (28). Acidification will also contribute to the potency of compounds such as, ROI, NO, and

peroxynitrite and increase their half-lives (56). Here we examine intracellular acidification of DC compared to macrophages. This may contribute to the inability of DC to *kill M. tuberculosis* and possibly other organisms.

Material & Methods

Mice

Adult female (8-10) week old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). NOS2-/- mice on a C57BL/6 background were generated by MacMicking, et al. (64) and breeding pairs were kindly provided by Dr. Timothy Billiar (University of Pittsburgh School of Medicine); mice were bred in our facility as homozygotes. B6.129S6-Cybb^{tm1} mice , (C57BL/6 background) have a genetic disruption of the p91 $^{phox-/-}$ gene in the NADPH complex (228), and were obtained from Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME). All mice were maintained in a specific pathogen-free Biosafety Level 3 facility.

Bacteria:

M. tuberculosis strain Erdman (obtained from the Trudeau Institute, Saranac Lake, NY) was passed through mice prior to freezing. Δ-icl-*M. tuberculosis* was derived from strain Erdman (229)and generously provided by Dr. John D. McKinney (Howard Hughes Medical Institute, Rockefeller University, New York, NY). *Mycobacterium smegmatis* strain MC^{2}155 was kindly provided by Dr. Graham Hatfull (Department of Biological Sciences, University of Pittsburgh), and BCG Tice strain obtained from Organon Teknika (Durham, NC). GFP-labeled *M. tuberculosis* strain Erdman was generously provided by Dr. Joel Ernst (University of California San Francisco School of Medicine, San Francisco, CA). These strains were grown in liquid (7H9 Middlebrook, Difco) medium, and frozen in aliquots at –80^{0}C. Aliquots were used to start

cultures at a concentration of 2.5 x 10^6 /ml in 7H9 medium; bacteria were grown in 5% CO_2 at 37°C. Cultures were used at day 6 or 7 to infect cells. The bacteria were washed and resuspended in DC or MØ media, sonicated 10s in a cup-horn sonicator, then added to the cell cultures after estimation of bacterial numbers based on previous experience. Enumeration of viable bacteria to confirm MOI was by plating for viable CFU on 7H10 Middlebrook medium and incubated for 18 days (3 days for *M. smegatis*) at 37 °C with 5% humified CO_2.

Culture and purification of DC and MØ

DCs and MØ were generated from the bone marrow cells of C57BL/6, NOS2-/-, and B6.129S6-Cybb^{tm1} p91 $^{phox-/-}$ mice. Briefly, cells were extracted from the femur and tibia bones of mice in DMEM medium. For the MØ cultures, cells were washed twice in DMEM and 2.5 X 10^6 cells were plated in LabTek PS petri dishes (Fisher Scientific, Pittsburgh PA) in 25 ml DMEM supplemented with 10% certified FBS, 1mM sodium pyruvate, 2mM L-glutamine (Life Technologies, Grand Island, NY), and 33% supernatant from L-cells fibroblasts cultured for 5-6 days. All reagents were LPS-free and no antibiotics were used. Medium was changed on day 3. On day 5, adherent cells were washed twice with ice-cold PBS (Life Technologies, Grand Island, NY), incubated for 20 minutes on ice, and harvested using cell scrapers (Becton Dickinson Labware, Lincoln Park, NJ). Cell concentration was adjusted to 1.0 x 10^6 cells/ml and cells were placed in Teflon jars (1 ml) (Savillex, Minnetonka, MN) or aliquoted into a 96 well plate (200µl/well) for infection. For DC cultures, bone marrow cells were centrifuged at 1200 rpm for 7 minutes, and red blood cells were lysed with NH_4Cl/Tris solution. T cells (CD4+ and CD8+) were removed using Low-Tox–M rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, NY) after incubation with anti-CD4 antibody (GK1.5 10µg/1 x 10^7 cells)

and anti-CD8 antibody hybridoma supernatant (anti-CD8α, clone 83−15−5). Cell concentration was adjusted to 1 x 10^6 cells/ml and adherent cells were depleted by overnight culture in DC medium containing DMEM, 2 mM L-glutamine, and heat inactivated 5% mouse serum (Sigma, St. Louis, MO). The non-adherent cells were cultured at 0.25 x 10^6 cells/ml in 24-well plates (Costar, Cambridge, MA) in DC medium containing 1000 Units/ml of rm-GM-CSF and rmIL-4 (Schering-Plough, Kenilworth, NH and kindly provided by Dr. Walter Storkus). At day 5 cells, non-adherent cells, were harvested, adjusted to 1.0 x 10^6 cells/ml in DC media containing rmGM-CSF (1000 U/ml) and either dispersed into a 24 well plate (1 ml/well) or aliquoted into a 96 well plate (200µl/well) for infection.

Infection of DCs and MØ

After culture for 5 days, DCs (at 1 x 10^6/ml in DC media + rmGM-CSF, without IL-4) were infected in 24 or 96 well plates with either *M. tuberculosis*, Δ-icl-*M. tuberculosis*, *M. smegmatis*, or *BCG* at an estimated MOI of 0.5-5. After 12 hours, unincorporated bacteria were removed by pelleting the DCs at low speed (<1000 rpm) and reculturing with fresh media. In some experiments, MØ were cultured and infected in 96 well plates; monolayers were washed to remove extracellular bacteria and fresh medium was added. In experiments involving quantitative cultures of intracellular bacterial growth, stasis or killing, the MOI was reduced to 1 and extracellular bacteria were removed after 4 hours, except for *M. smegmatis* where we used a lower MOI and removed bacteria at 1 hour post-infection. To estimate the percentage of infected cells for each experiment, DCs and MØ were either airdried on poly-L-lysine coated slides or grown in parallel in glass culture well slides (Nalgene) and fixed in 1% paraformaldehyde at

each time point. Slides were stained by the Kinyoun method for acid–fast bacteria. For phenotypic assays, DCs and MØ were cultured for an additional 48 hours.

Reactive Oxygen Intermediate Assay

Day 5 bone-marrow derived DC and MØ (1×10^6) cells per ml were activated with 250 units/ml IFN-γ overnight and 1 μg/ml LPS. The assay was performed as described in (230). Briefly, cells were washed twice with Kreb's Ringer Phosphate Buffer with Glucose (KRPG) (15.6 mM Na Phospahte Buffer, 120 mM NaCl, 4.8 mM KCl, 0.54 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 11mM Glucose), resuspended in KRPG at 2×10^6 cells per ml, and then 4×10^5 were placed in a cuvette with 250 μg ferrricytochrome C, 2.5 μg catalase, and 0.25 μg PMA. The control sample contained 12 μg/μl SOD to inhibit the reduction of ferrricytochrome C (Sigma, St. Louis). Readings were taken at 550 nm every 30 seconds for 1 hour on a 14DS UV/visible spectrometer (Aviv instrument, New Jersey).

Peroxynitrite assay

Day 5 bone-marrow derived DC and MØ were activated with 250 units/ml mouse recombinant IFN-γ overnight and 1 μg/ml LPS. Peroxynitrite assay was performed essentially as described in (68). Briefly, the activated cells were then stimulated with 400 ng PMA and incubated in Hank's balanced salt solution at 4×10^6 cells in 24-well plates in the presence of 1mM 4-hydroxyphenlacetic acid (4-HPA) with or without Cu, Zn superoxide dismutase (0.1 μg/ml) (Oxis Research, Eugene, OR) for 4 hr at 37°C. Supernatants were filtered (0.45 μM filters) and assayed for NO_2-HPA at 420nm on a 14DS UV/visible spectrometer (Aviv

instrument, New Jersey). Peroxynitrite concentrations were determined using the conversions for NO_2-HPA reported in (68).

Determination of nitrite accumulation:

Nitrite (NO_2^-) accumulation in the supernatant of cultured cells was measured as an indicator of total RNI production by a Griess Assay, with a sodium nitrite standard, as previously described (208)(Chapter 2). Supernatants from 2 x 10^5 cells (100 μl) of each condition were assayed in duplicate or triplicate and absorbency was measured at 570 nm using an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA).

Intracellular growth for mycobacteria

For determination of actual intracellular colony forming units (CFU), DCs and MØ cultures were prepared and activated as above, and cell lysates (lysed in 0.1% saponin) at each time point were cultured on 7H10 plates (10-fold dilutions in PBS + 0.05% Tween). The number of extracellular bacteria was determined by plating the undiluted sonicated supernatant of each timepoint. The number of initial intracellular bacteria was determined at 1 or 4 hours post-infection (*M. smegmatis* and BCG, or *M. tuberculosis*, respectively), and reduction of input was based on that number. CFU were counted after incubation of plates at 37°C for 3 days for *M. smegmatis*) and 18 days for *BCG and M. tuberculosis*.

RNAse protection assay (RPA)

Determination of the levels of mRNA for the genes of interest at various time intervals after the infection was performed using a multiprobe RNAse protection assay (Pharmingen, San

Diego, CA). Total RNA was extracted from DC and MØ, cultured and uninfected or infected with *M. tuberculosis* as detailed before, using Trizol reagent (Life Technology, Grand Island, NY). The extracted RNA was subjected to RPA according to manufacturer's instructions. Protected [^{32}P]UTP-labeled probes were resolved on a 6% urea polyacrylamide gel and analyzed by autoradiography. Cytokine analysis was performed using a custom made probe set (Mck3) specific for NOS2, IL-12p40, IL-1α, IL-1β, IFN–γ, and IL-10. The expression of specific genes was quantified densitometrically (Image Quant, Molecular Dynamics, Sunnyvale, CA) relative to the abundance of housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or L32.

Western Blot Analysis

DCs and MØ, uninfected or infected (MOI 3) as described above, were plated in 24 well plates at 1 x 10^6 cells/well. Four hours post infection, DC were collected and centrifuged at 1200 RPM for 7 minutes, and supernatant was retained. Supernatants were collected from MØ and both supernatants were used in Griess Assays. 250 µl of Western lysis buffer [1X TBS, 1% Nonidet P-40 (Pierce, Rockford, IL), protease inhibitor cocktail (10 µM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, Sigma, St. Louis, MO)] was added to lyse the cell pellets (DC) or cell monolayer (MØ). Lysates were heated at 90°C for 15 minutes to kill mycobacteria. Lysates then were incubated on ice for 20-30 minutes, cellular debris was pelleted out for 15 minutes at 14,000 rpm at 4^{o}C. Protein concentration was determined by Bio-Rad D_C assay per manufacturer's instructions (BioRad, Hercules, CA). 40 µg protein were added to an equal volume of 1X sample buffer (100 mM Tris pH6.8, 0.2%, bromophenol blue, 20 % glycerol, 4 % SDS, 200 mM dithiothreitol), boiled 5 minutes, then separated on both 7.5% and

12.5 % SDS-PAGE gels (Running buffer: 25mM Tris, 192mM glycine, 0.1% SDS) and either stained with Coomassie Brilliant Blue (Sigma, St. Louis, MO) or transferred to nitrocellulose (Micron Separations Inc., Westboro, MA) at 100V for 1 hour at 4^{o}C in Western transfer buffer (25 mM Tris, 192 mM glycine, and 20% Methanol, pH 8.3). The nitrocellulose membrane was stained with Ponceau S (Sigma, St. Louis, MO) to visualize transferred proteins, rinsed with de-ionized water, then blocked 1 hr at room temperature with either 3% blocking reagent (Upstate Biotechnologies, Lake Placid, NY) in PBS (for anti-nitrotyrosine Ab) or 5% milk in TBS (for anti-NOS2 Ab) while gently rocking. The membrane was washed twice with TBS+ 0.1% Tween 20 (Sigma, St. Louis), then anti-NOS2 antibody (clone 2) (Transduction Laboratories, San Diego, CA) was added at 1:2000 dilution (in 20 ml of 5% milk in TBS + 0.1% Tween 20) or anti-nitrotyrosine antibody at 2µg/ml [in 10 ml of 3% Blocking Reagent (Upstate Biotechnologies, Lake Placid, NY) in PBS] for 1 hour at room temperature while gently rocking. The membrane was washed with TBS/0.1% Tween-20, and goat anti-rabbit IgG peroxidase conjugated secondary antibody (Sigma, St. Louis, MO) was added at 1:5000 for 1 hour at RT. The membrane was washed, then ECL (Amersham Lifescience, Piscataway, NJ) and autoradiography were used to visualize anti-NOS2 and anti-Nitrotyrosine reactive proteins. Molecular weights for proteins were determined from the pre-stained broad range MW standards (Bio-Rad, Hercules, CA) which were run on the gels.

Controls include nitrotyrosine immunoblotting control (Upstate Biotechnology, Lake Placid, NY) which consists of nitrated bovine superdismutase (SOD, ~16 kDa), nitrated bovine serum albumin (BSA, ~66 kDa), and nitrated rabbit muscle myosin (~215 kDa); and mouse macrophage lysate (Transduction Laboratories, San Diego, CA) which contains 130 kDa NOS2.

Immunoprecipitation

Bone-marrow derived macrophages were cultured as described above. At day 5 the medium was removed, cells were washed twice with warm PBS, and DMEM without methionine and cysteine plus 10 µM HEPES and 2mM glutamine was added for 1 hr at 37^{o}C with 5% CO_2 humidification. Cells were then labeled with [^{35}S]-methionine at 100 µCi/ml for 2.5 hrs in a volume of 2 ml. Cells were washed with PBS, lysed with Western lysis buffer (see above), and heated at 90 ^{o}C for 15 minutes to kill the *M. tuberculosis*. Lysates were incubated on ice for 30 minutes and transferred to a microfuge tube and spun at 13,000 rpm at 4 ^{o}C; supernatants were collected. 5µl of a 1:20 dilution of the polyclonal anti-nitrotyrosine (Upstate Biotechnologies, Lake Placid, NY) or anti-NOS2 antibody (Transduction Laboratories, San Diego, CA) was added to 95 µl of lysates and incubated on ice for 4hr, then an equal volume of 20% protein A-agarose Sepharose beads (BioRad, Hercules, CA) in lysis buffer was added, samples were vortexed for 30 mins, pelleted, washed 3 times in lysis buffer, resuspended in 30 µl of 1X sample buffer (100 mM Tris pH6.8, 0.2%, bromophenol blue, 20 % glycerol, 4 % SDS, 200 mM dithiothreitol (DTT)), boiled 5 minutes to elute proteins The labeled proteins were separated on 7.5 and 12.5% SDS-PAGE gels with pre-stained molecular weight standards (Bio-Rad, Hercules, CA). The gel was treated with Amplify (Amersham Pharmacia Biotech, UK) for 30 minutes, dried under vacuum at 80 ^{o}C, exposed to X-ray film.

Intracellular Acidification

The acidotropic dye Lysotracker Red DND-99 (Molecular Probes, Eugene, OR) was diluted in DMEM (1:10,000) for co-localization experiments with GFP-labeled *M. tuberculosis* (kindly provided by Dr. Joel Ernst, University of San Francisco, CA). Bone-marrow derived DC

or macrophages were cultured in glass culture well slides (Nalgene) at 2 X 10^5 cells/well. The cells were pre-loaded with lysotracker for 2 hrs prior to activation and infection with GFP-*M. tuberculosis* as described above. When extracellular bacteria were removed 4 hr post-infection fresh media and lysotracker was added overnight, then the cells were washed in PBS, fixed in 1% PFA, and mounted with coverslips using Gelvatol or Slow Fade (Molecular Probes, Eugene, OR). Images were obtained using a Leica TCS NT Confocal Scanning Microscope over 5 fields per sample were viewed.

Statistics

For statistical analysis of samples, paired and unpaired student t tests were used (Instat, v. 2.03, GraphPad Software, San Diego, CA and StatView, Abacus Concepts, Berkeley, CA). P values <0.05 were considered significant.

Results

Expression of NOS2 mRNA in DC and macrophages after infection with *M. tuberculosis*.

The message for NOS2 was examined in untreated or IFN–γ and LPS activated *M. tuberculosis*-infected-DC and macrophages by RPA using a template for NOS2 to determine whether there were differences at 4 hours (Figure 17). We have previously reported that at 4 hr post-infection in some, but not all, experiments, a detectable difference in nitrite levels after infection was observed (Chapter 2). Macrophages usually produced more nitrite at this timepoint, although in some experiments DC production of nitrite was higher (see Table 3). It was possible that this early burst of RNI production contributed to the ability of MØ to kill *M. tuberculosis*. However, even in experiments where DC produced more nitrite at the 4 hr

timepoint, the intracellular bacilli were still not killed (Table 3). Despite similar levels of RNI production by IFN-γ and LPS-activated *M. tuberculosis*-infected DC and macrophages, there was a difference in the intracellular killing of *M. tuberculosis* (23). DC were unable to kill intracellular *M. tuberculosis*, yet controlled the growth by a NOS2 dependent mechanism. In contrast, activated macrophages could kill 50% of all intracellular *M. tuberculosis* by a NOS2 dependent mechanism.

NOS2 is the enzyme necessary for the production of nitric oxide via the arginine pathway and therefore it was of interest to compare the mRNA for this enzyme in DC and macrophages. The activated infected DC actually had higher levels of NOS2 mRNA at 4hr, whereas at other timepoints the levels were similar to, or slightly lower (24hr) than the NOS2 mRNA expressed in macrophages (Figure 17). Regardless of the higher levels of NOS2, the activated DC do not usually produce more nitrite in response to activation than the macrophages, and they were consistently incapable of killing the intracellular *M. tuberculosis* (Table 3).

Expression of NOS2 protein in DC and macrophages early in *M. tuberculosis* infection.

We used Western Blot analysis to examine NOS2 on the protein level to determine if the higher expression mRNA at 4 hrs in DC resulted in more protein expression (Figure 18). We examined lysates from uninfected, *M. tuberculosis*-infected, IFN-γ and LPS-activated *M. tuberculosis*-infected DC and macrophages over a timecourse of 72 hours post-infection. Western blot of DC and MØ proteins demonstrated that the amount of NOS2 protein was higher in activated DC than in activated MØ at the 4 hr time point (Figure 18), as well at later time points (data not shown). Although there is more NOS2 protein in the DC, the output of nitrite is higher in macrophages cultures (Figure 18B). NOS2 protein may not be fully functional in DC

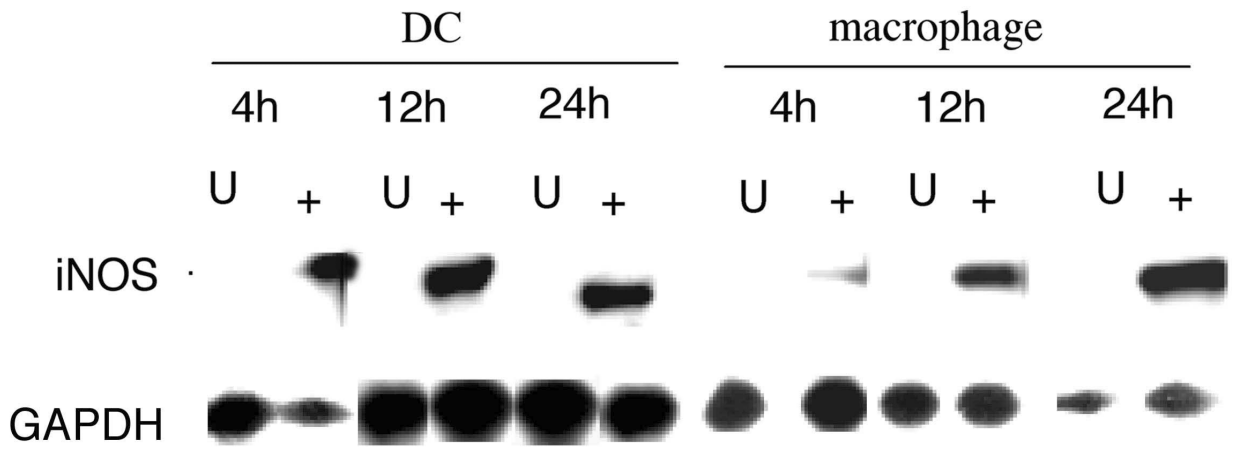

Figure 17. Expression of NOS2 mRNA in DC and macrophages after infection with *M. tuberculosis.*

RNA from uninfected or *M. tuberculosis*-infected unactivated (U) or IFNγ/LPS activated (+) DC and macrophages at different time points post-infection was incubated with a ^{32}-[P] labeled probe made from the custom designed mck-3 template as directed by the manufacture (Pharmingen, NJ). (U) unactivated *M. tuberculosis*-infected; (+) IFNγ/LPS activated *M. tuberculosis*-infected cells. Levels of NOS2 were standardized to the levels of GAPDH by densitometry (data not shown).

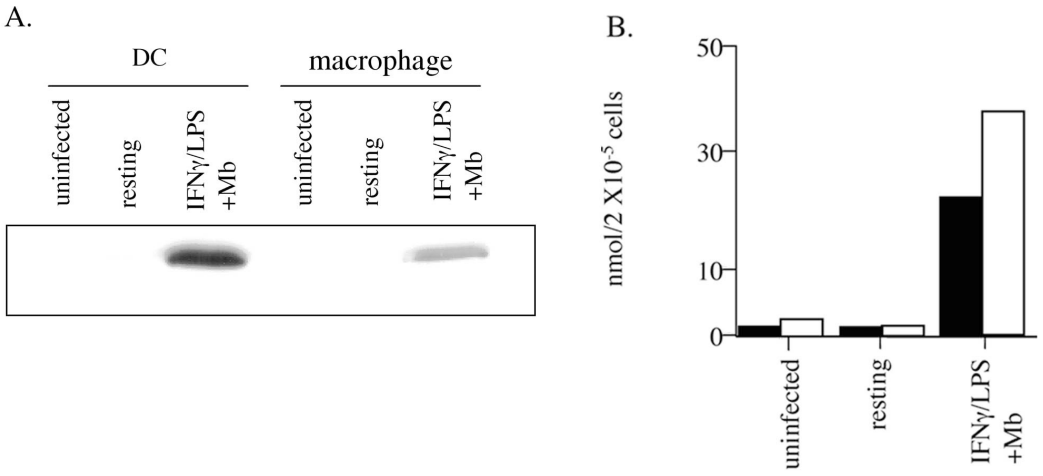

Figure 18. Expression of NOS2 protein in DC and macrophages early in *M. tuberculosis* infection.

A. Cell lysates of either uninfected, untreated *M.tuberculosis*-infected, IFN-γ and LPS activated *M. tuberculosis*-infected bone-marrow derived DC and macrophages were collected 4 hours post-infection and separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with milk, and then incubated with an antibody directed against NOS2 (Upstate Biotechnologies, NY) and a secondary antibody conjugated with horseradish peroxidase (Sigma, St. Louis). Bands were visualized by ECL chemilluminescence (Amersham Lifescience, Piscataway, NJ).

B. Supernatants from these cells were tested for nitrite production by a Greiss assay. Black bars, DC; white bar, macrophage.

IFN-γ/LPS MØ		IFN-γ/LPS DC	
*nmol nitrite	[b] fold change	nmol nitrite	fold change
105	-1.9	37.5	+1.2
27	-1.9	109	+0.8
50	-1.9	25	+1.5
20	-1.2	0	+2.0
1.9	-1.2	19	+1.5
1.9	-1.2	19	+1.5

Table 3. Differences in nitrite production by activated DC and MØ.

*Supernatants from murine bone-marrow derived DC and MØ with IFN-γ and LPS and infected with *M. tuberculosis* were examined by a Greiss Assay for nitrite production (as detailed in Materials & Methods).

*The standard curve was generated using $NaNO_2$.

[b] fold change of intracellular CFU at 48 hours compared to the intracellular CFU at 4 hours (input).

compared to macrophages, due to unique post-translational modification or availability of substrate, arginine. These data indicate that although RNI production is necessary for the intracellular bacteriostatic and bactericidal effects on *M. tuberculosis*, inadequate NOS2 expression or total RNI production do not appear to be the underlying reason for inability of DC to kill intracellular *M. tuberculosis*.

DC are capable of producing larger quantities of reactive oxygen intermediates (ROI).

The production of ROI by DC has not been investigated fully. Human-derived DC were shown to produce ROI in response to a *Candida albicans* infection (195). To explore an underlying mechanism for the inability of DC to kill *M. tuberculosis* despite similar RNI production as activated MØ, we investigated production of ROI. ROI are a known anti-microbial mechanism, typically used by macrophages to kill intracellular pathogens, although the role in killing *M. tuberculosis* is controversial. ROI concentration was measured using an assay based on the reduction of ferricytochrome C (230). Surprisingly, we observed that IFN-γ+LPS-activated DC stimulated with phorbol 12-myristate 13-acetate (PMA) produced larger quantities of ROI than IFN-γ+LPS-activated MØ stimulated with PMA (Figure 19A). ROI and RNI are triggered by different stimulus in macrophages. Stimulation of these cells with PMA is typical and necessary in this assay to enhance the ROI production which, has a very low absorbency reading; it does not alter the pattern of production (230).

Since the effect of ROI on *M. tuberculosis* is not well understood, the higher production of ROI from DC does not necessarily indicate a more potent anti-microbial effect against *M. tuberculosis* for DC. We examined the production of nitrites in the supernatants of these samples

(Figure 19B) and observed that despite difference in the ROI production the nitrite production was similar between activated DC and macrophages.

Activated DC produced more peroxynitrite than activated MØ.

Peroxynitrite ($ONNO^-$) is formed by the interaction of nitric oxide (NO) and superanion (O_2^-) as represented in the equation:

$$O_2^- + NO^- \rightarrow ONNO^-$$

Increased production of ROI may lead to increased $ONOO^-$ as it is the limiting factor in the production of peroxynitrite (68). Peroxynitrite is a strong oxidant and has the ability to kill many microorganisms, yet direct evidence of peroxynitrite killing *M. tuberculosis* has never been shown. In fact, a recent publication provided evidence that peroxynitrite is not toxic to *M. tuberculosis*, although other RNI can kill this organism (49). We compared the amount of peroxynitrite production by activated DC and MØ using a spectrometeric assay that measures the Cu, Zn superoxide dismutase nitration of 4-hydroxyphenlacetic acid (4-HPA) to 4-NO_2HPA by peroxynitrite in the supernatants of cells (68). Activated DC produced approximately 2-fold more peroxynitrite than activated MØ both uninfected (Figure 20A) and infected with *Mycobacterium smegmatis* (Figure 20B), a non-pathogenic species of mycobacteria which does not require Biosafety Level 3 containment and can be used in this assay.

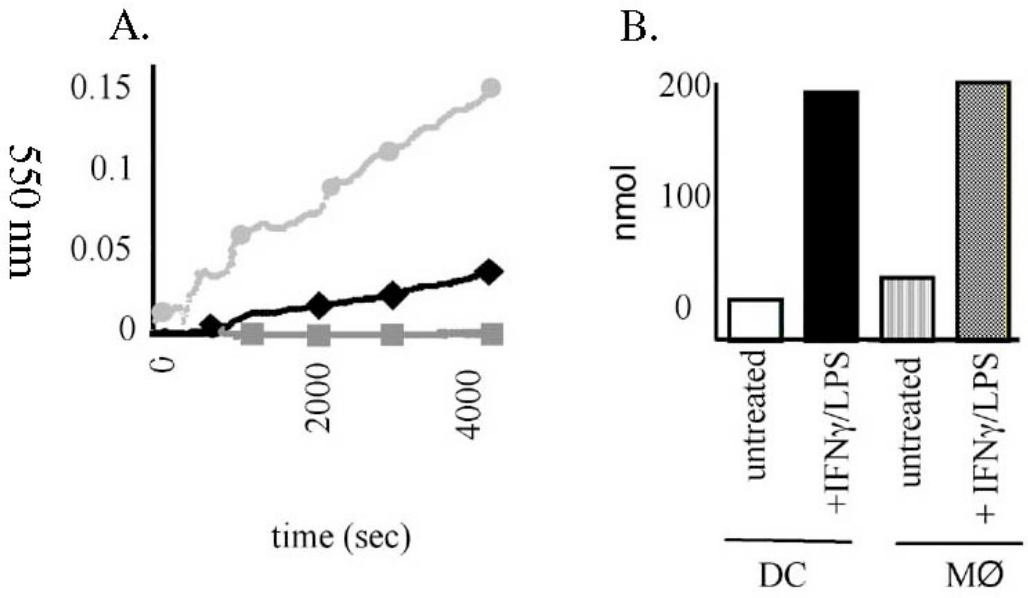

Figure 19. Activated DC are capable of producing larger quantities of ROI than activated MØ.

A. ROI production was quantitated by assaying the reduction of ferricytochrome C at 550 nm on a UV vis spectrometer as described in the Material & Methods. Activated DC (light gray line with circles); activated MØ (black line with diamonds); controls: activated DC and MØ with SOD (gray line with squares). (230) **B**. Nitrite (NO_2-) accumulation from the supernatant of these cultured cells (4 X 10^5 cells) was measured as an indicator of NO production by a Greiss Assay, with a sodium nitrite standards, as previously described (208).

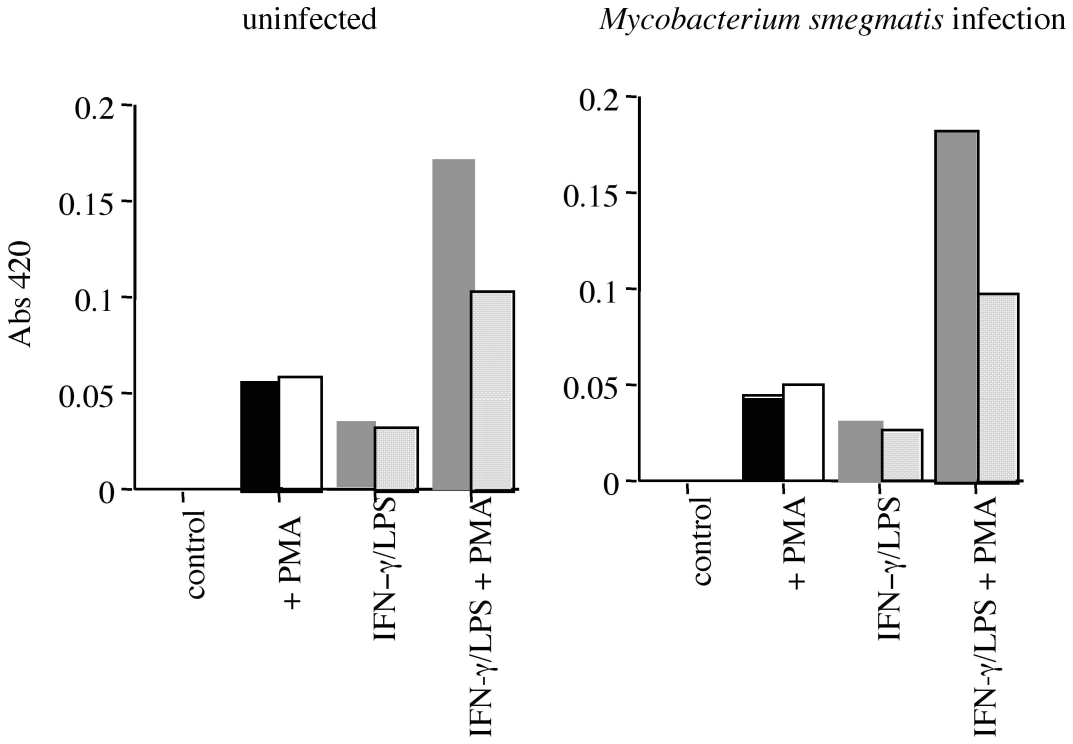

Figure 20. Activated DC produced more peroxynitrite than activated MØ.

(**A**) Untreated or (**B**) *Mycobacterium smegmatis* infected DC (black) and macrophages (white) or activated DC (gray) and macrophages (light gray) were cultured and activated as previously described then incubated with 4-hydroxyphenlacetic (4-HPA) for 4 hours in the absence or presence of PMA. Cu, Zn superoxide dismutase was added to these cells, the presence of peroxynitrite production in the supernatant will cause nitration of 4HPA resulting in the production of NO_2-HPA which is assayed on a dual beam spectrometer at 420nm (68).

Nitrosylation of proteins did not occur in *M. tuberculosis* infected IFN-γ and LPS activated DC and macrophages within 72 hours.

We attempted confirmation of peroxynitrite production *in vivo* by examining nitrosylation of protein within lysates of activated infected DC and macrophages. Nitrosylation of proteins occur when peroxynitrite is present, and is referred to as a footprint of peroxynitrite. The nitration of tyrosine residues can render protein and enzymes inactivate (56). In chronic infections, nitrosylated proteins are readily detectable as there has been sustained peroxynitrite production (231). Nitrosylated proteins were easily detected in the lungs of mice infected with *M. tuberculosis*, where NOS2 expression is high (232)**.** We used Western blot analysis with an anti-nitrotyrosine antibody to examine nitrosylated proteins in activated and infected macrophages at 4, 24, and 48 hrs post-infection and activated DC 4 hr post-infection (Figure 21, top). We were consistently unable to detect the presence of any nitrosylated proteins in these lysates, despite high levels of proteins transferred to the membrane (observed after staining with a protein dye, Ponseau S). A low amount of a positive control containing nitrosylated bovine superdismutase, nitrated bovine serum albumin, and nitrated rabbit muscle myosin, was readily detectable with the anti-nitrotyrosine antibody. These same lysates did cross-react with anti-NOS2 antibody, indicating that the cells were activated and expressed enzyme (Figure 21, bottom). At all timepoints there were detectable levels of NOS2 (130 kDa) (control indicated) and this matched the data from the Greiss assay showing that these cells were producing nitrite (data not shown).

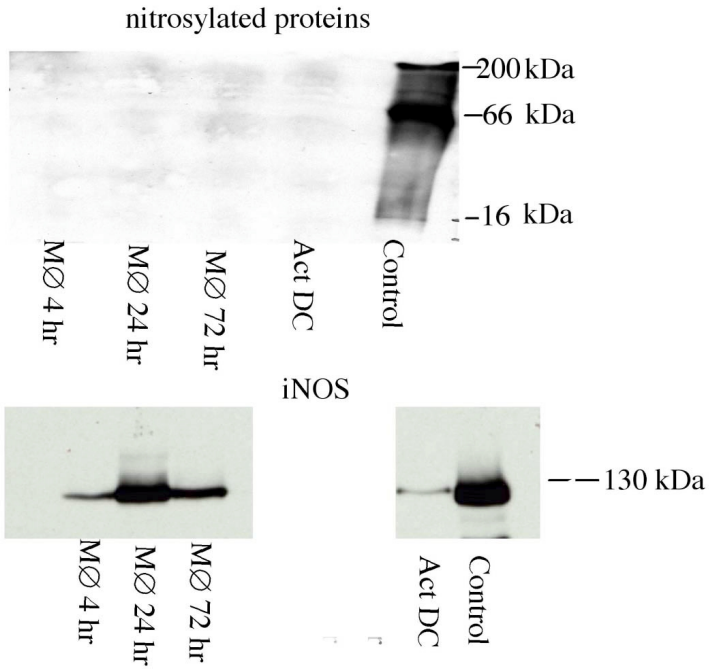

Figure 21. Nitrosylation of proteins did not occur in *M. tuberculosis*-infected, IFN-γ and LPS activated DC and macrophages within 72 hours

.

Cell lysates of IFN-γ and LPS activated *M. tuberculosis*-infected bone-marrow derived DC (4 hr post-infection) and macrophages (4, 24, and 72 hr post-infection) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with de-nitrosylated milk (Upstate Biotechnology, NY), and then incubated with an antibody directed against nitrotyrosine (top) or NOS2 (bottom) (UpstateBiotechnologies, NY) and a secondary antibody conjugated with horseradish peroxidase (Sigma, St. Louis). Bands were visualized by ECL chemilluminescence (Amersham Lifescience, Piscataway, NJ). Controls: top, nitrosylated proteins: rabbit muscle myosin (215 kDa), BSA (66kDa), SOD (16 kDa) (Upstate Biotechnologies, NY); bottom, NOS2 protein (130kDa) (Upstate Biotechnologies, NY).

Nitrosylated proteins could not be immunoprecipitated from the IFN-γ and LPS-activated *M. tuberculosis*-infected DC and macrophages.

We used a more sensitive and specific technique in a further attempt to detect nitrosylated proteins within the lysates of activated infected DC and macrophages. We radiolabeled IFN-γ and LPS-activated, *M. tuberculosis*-infected DC and macrophages with ^{35}S-Met and performed immunoprecipitations using anti-nitrotyrosine antibody. The immunoprecipitation technique allows for the extraction of the proteins bound to the antibody, in this case nitrosylated proteins, from the total pool of proteins; these were separated by SDS-PAGE and visualized by autoradiography. NOS2, which is expressed in these cells (Figure 22) was used as a positive control. Total radiolabeled lysates were run to visualize the amount of metabolic labeling. A very long exposure of several weeks was required to detect the radiolabeled lysates, suggesting that the metabolic labeling of these cells was poor. NOS2 was observed in all timepoints (130Kda), but there was no detectable nitrosylation. We believe that the inefficient labeling may have hampered the detection of these proteins. This may be because the cells used were primary cells and not actively dividing, rather than a cell line. Alternatively, early in infection there may not be detectable levels of nitrosylated proteins since the half live of peroxynitrite is extremely short (1s) and there may not be sufficient chronic exposure to peroxynitrite to nitrosylate proteins.

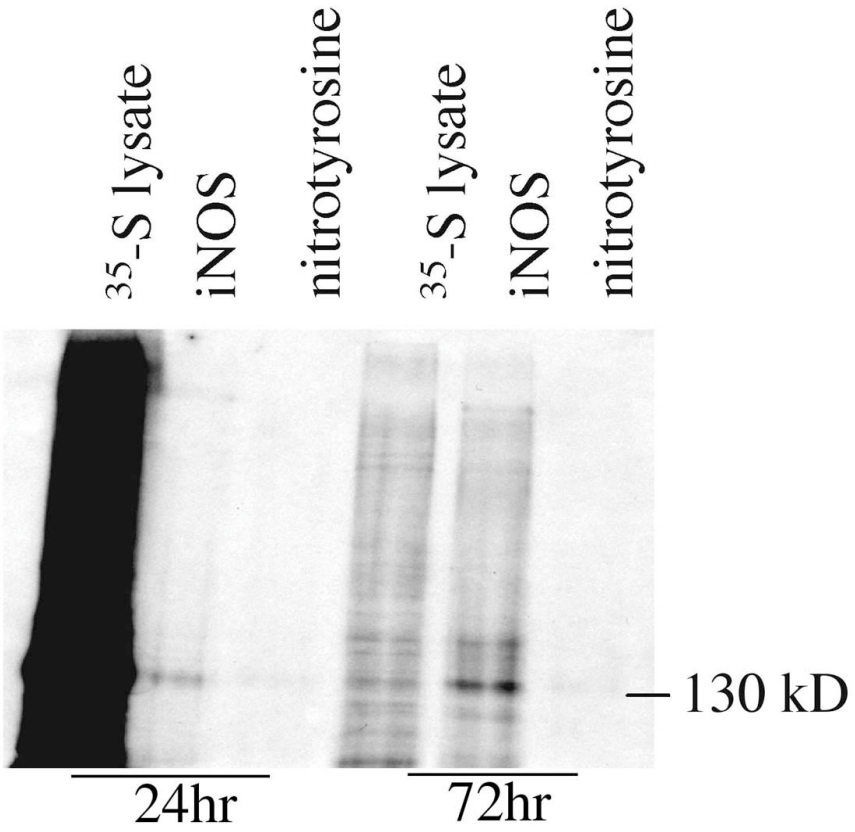

Figure 22. Nitrosylated proteins could not be immunoprecipitated from the IFN-γ and LPS-activated *M. tuberculosis*-infected DC and macrophages.

Activated *M. tuberculosis*-infected DC and macrophages were incubated in Met-free media then labeled with ^{35}[S]-Met. Lysates were prepared and incubated with antibody recognizing nitrosylated proteins or NOS2 (positive control) (Upstate Biotechnology, NY), then incubated with Protein A sepharose beads. Beads were repeatedly washed, and the proteins were eluted by boiling for 10 minutes, and separated by SDS-PAGE. NOS2 has a MW of 130kDA, which is indicated by a line. ^{35}[S]- lysates were not incubated with antibody and represent total labeled proteins.

Mycobacterium smegmatis and *Bacillus –Calmette Guerin (BCG)* are killed within activated DC.

Mycobacterium smegmatis and BCG have high or moderate susceptibility, respectively, to peroxynitite (49). We tested the ability of activated DC, which produced more peroxynitrite than activated macrophages, to kill these susceptible mycobacterial species. We observed that the intracellular bacterial numbers for both *M. smegmatis* (Figure 23) and BCG (Figure 24) were reduced below the input number of these bacteria in not only the activated MØ but the activated DC as well, suggesting that these bacilli are being killed. This differs from the results for virulent *M. tuberculosis* which was not killed by DC, possibly because virulent *M. tuberculosis* is not very susceptible to peroxynitrite. In activated DC there was a 25% reduction of *M. smegmatis* at 24 hours post-infection (p.i.) then 30% reduction by 48 hours p.i.; this was comparable to macrophages where there was a 40% reduction at 24 hours p.i. and 27% at 48 hours. In activated DC there was a 15% reduction of BCG at 24 hours p.i. and 43% reduction by 48 hours p.i.; in activated macrophages infected with BCG there was a 29% reduction at 24 hour pi then only a 15% reduction by 48 hr. In a few experiments the activated DC infected with BCG had 1.5 - 3 fold difference in killing (compared to activated macrophages) possibly related to more peroxynitrite production. Importantly, in activated *M. tuberculosis*-infected DC no reduction was observed, in contrast to the results obtained with these avirulent mycobacterial species. *M. tuberculosis* was reported to be resistant to peroxynitrite whereas *M. smegmatis* and *BCG* were susceptible to peroxynitrite (49). In activated macrophages infected with *M. tuberculosis* there is approximately 50% killing, we probably see less in the *M. smegmatis* and

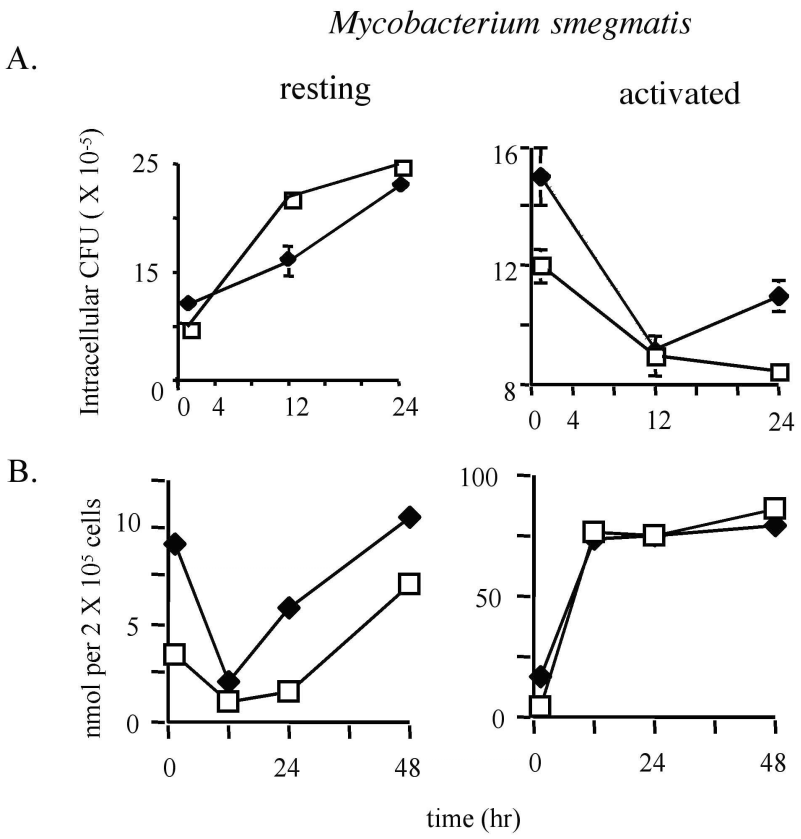

Figure 23. *Mycobacterium smegmatis* are killed within activated DC.

A. Cell pellet lysates of *M. smegmatis*-infected DC (black diamonds) and MØ (white squares) (MOI=1), resting or activated with IFN-γ + LPS, were serially diluted in PBS + 0.05% Tween 80 and plated on 7H10 plates, which were incubated for 3 days at 37°C and 5% CO_2. Three wells per condition were assessed at each timepoint, and the mean intracellular CFU values are reported at various time points after infection of DC and MØ. At each timepoint, <1% of total CFU was found in the supernatant. A representative of two experiments is shown. Error bars show standard error. **B.** Nitrite (NO_2-) accumulation in the supernatant of the infected cells was measured by Greiss Assay, with a sodium nitrite standards, as previously described (2).

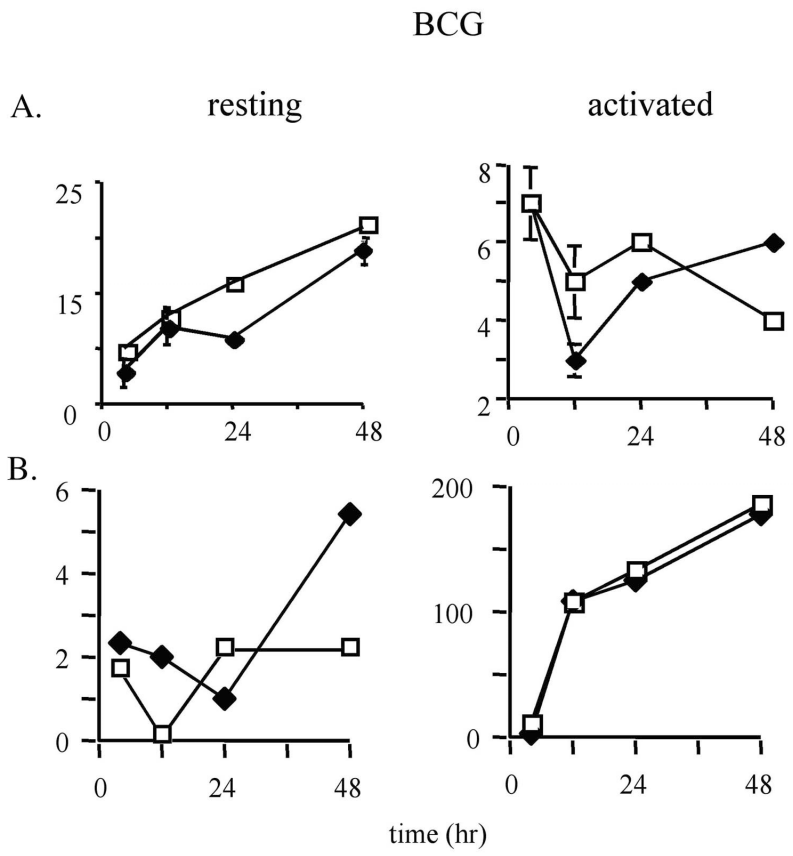

Figure 24. *BCG* is killed within activated DC.

(**A**.) Cell pellet lysates of *BCG*-infected DC (black diamonds) and MØ (white squares) (MOI=1), unactivated or activated with IFN-γ + LPS, were serially diluted in PBS + 0.05% Tween 80 and plated on 7H10 plates, which were incubated for 18 days at 37°C and 5% CO_2. Three wells per condition were assessed at each timepoint, and the mean intracellular CFU values are reported at various time points after infection of DC and MØ. At each timepoint, <1% of total CFU was found in the supernatant. A representative of two experiments is shown. Error bars show standard error. (**B**.) Nitrite (NO_2-) accumulation in the supernatant was measured by Greiss Assay, with a sodium nitrite standards, as previously described (2). DC (black diamonds) and MØ (white squares).

BCG infection since the bacterial burden was 100-150 times higher. The amount of nitrite in the supernatants of these cultured cells was comparable (as measured by Greiss Assay)(Fig. 23B, 24B). There was little to no RNI produced by the resting cells.

M. tuberculosis growth within activated DC and macrophages from reactive oxygen intermediates (ROI)-deficient mice.

We observed the growth of *M. tuberculosis* in DC and macrophages obtained from B6.129S6-Cybb^{tm1} (phox-/-) mice in which the gene encoding the 91 kD subunit of oxidase cytochrome b (p91*phox*) was disrupted (Jackson Laboratories, Bar Harbor,and disrupts the production ROI (228). This mouse is a model for Chronic Granulomatous Disease. It was reported that infection of these mice with *M. tuberculosis* led to initially higher bacterial burdens, although at later time points, CFU were similar to wild type mice (216). The deficiency in ROI production in the p91$^{phox-/-}$ mice severely limits the amount of peroxynitrite that can form, although other RNI are present (Figure 26). In the activated DC from p91$^{phox-/-}$ mice there was a reduction of bacilli below the input level at 4 hr (Figure 25B). In contrast, in the wild-type DC (Figure 25A) as reported previously, the bacteria were not able to grow but were not killed.

Unexpectedly, in the activated macrophages we observed a reversal of killing in the p91$^{phox-/-}$ mice. The bacteria were not growing as readily as in the resting macrophages (compare Figure 25 B to A) but there was no reduction in the intracellular bacilli as compared to the input (4hr); indeed there may even a slight increase in bacteria. This suggests a role for ROI in killing of *M. tuberculosis* within activated macrophages, and this role would appear not to be dependent on the formation of peroxynitrite. Since there is uncompromised total RNI nitrite production by

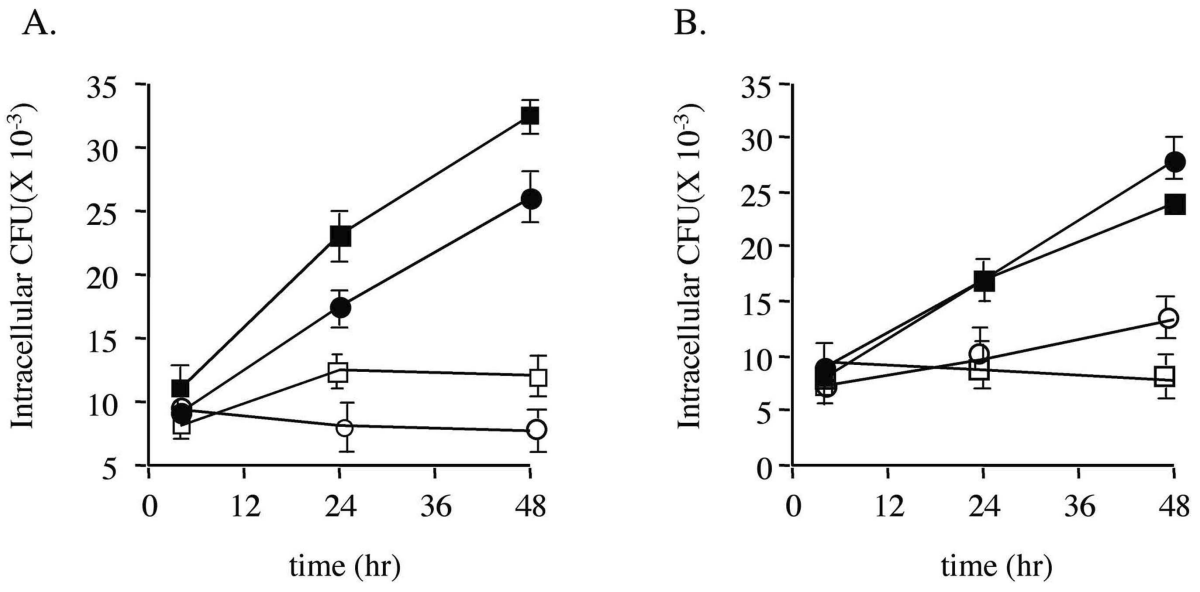

Figure 25. *M. tuberculosis* growth within activated DC and macrophage from ROI-deficient mice.

Cell pellet lysates of *M.tuberculosis*-infected DC (squares) and MØ (circles) (MOI=1) isolated from the bone-marrow of (**A**.) C57Bl6 mice (**B**.) p91 $^{phox-/-}$ mice (Jackson Laboratory, ME), were left unactivated (filled) or activated (open) with IFN-γ-LPS, then serially diluted in PBS plus 0.05% Tween 80 and plated on 7H10 plates, which were incubated for 18 days at 37°C and 5% CO_2. Three wells per condition were assessed at each timepoint, and the mean intracellular CFU values are reported at various time points after infection. DC (squares) and MØ (circles). At the input 4 hr timepoint <1% of total CFU was found in the supernatant. A representative of three experiments is shown. Error bars show standard error.

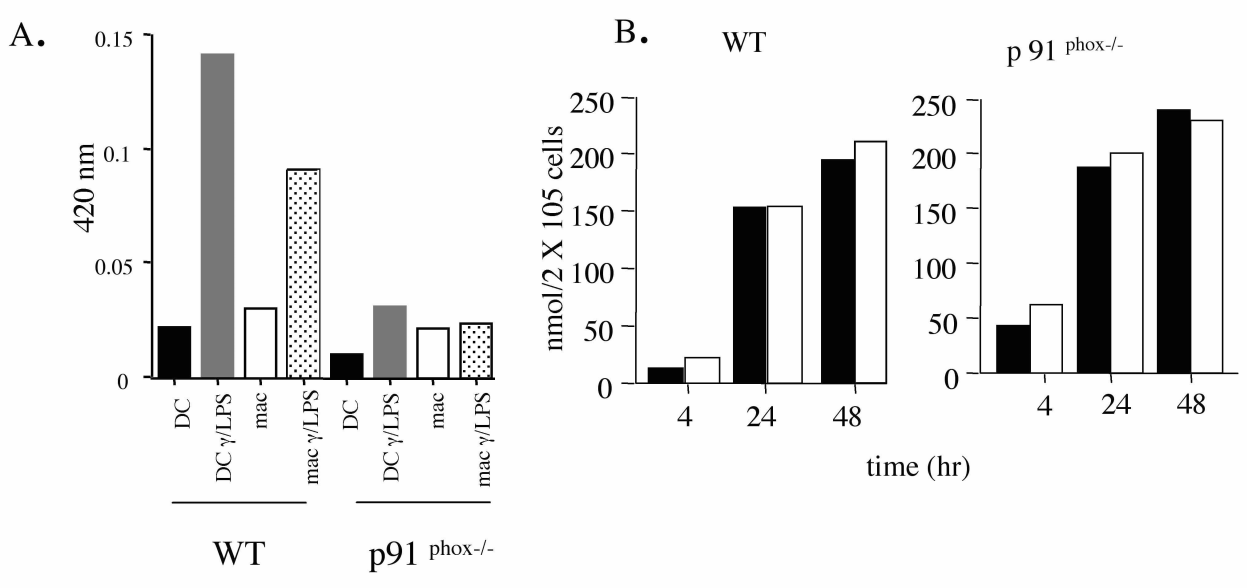

Figure 26. Activated DC and activated MØ from p91$^{phox-/-}$ mice did not produce substantial amounts of peroxynitrite.

(A.) Untreated DC (black) and MØ (white) and IFN-γ/LPS activated DC (gray) and macrophages (dots) from WT and p91^{phox} mice were cultured and activated as previously described. The peroxynitrite production was measure using an assay previously described in Material and Methods and (68). **(B)**. Griess assay were performed on the supernatants from these IFN-γ/LPS activated DC (black) and macrophages (white) derived from WT and p91^{phox} mice and read at 570 nm. The standard curve was generated using $NaNO_2$

the p91$^{phox-/-}$ cells, as compared to wild type (see Figure 26), this suggests that the macrophages may need ROI to efficiently use the RNI, or that ROI may have their own role. Yet it does appear that activated DC benefit from the lack of ROI since the RNI available in the absence of ROI appear to kill intracellular *M.tuberculosis.*

M. tuberculosis growth within activated DC and macrophage from RNI-deficient mice. To establish the potentially different contribution of ROI in activated DC and macrophages to controlling the intracellular growth of *M. tuberculosis*, we examined the growth of the bacteria within RNI-deficient mice (NOS2-/-) (Figure27). In this system the RNI are eliminated from playing a role in killing of the bacteria although ROI are still present. Mice with the gene for NOS2 disrupted are very susceptible *to M. tuberculosis,* highlighting the necessity of RNI in control of infection (64) [Scanga in press]. In untreated NOS2-/- DC and macrophages, the bacteria grew readily. Upon activation, the NOS2-/- DC and macrophages were deficient in inhibition of growth and killing the intracellular *M. tuberculosis*. This was expected, and agrees with our previous data, since RNI production was impaired. The ROI alone were not sufficient to inhibit the growth of the bacteria in the cells.

Acidification within activated DC and macrophages.

The apparent lack of effectiveness of RNI, peroxynitrite, and ROI in killing *M. tuberculosis* within activated DC could be due to a microenvironment (e.g. the phagosome or phagolysosome) that is not sufficiently acidic. It has been demonstrated that *M. tuberculosis* and some other mycobacteria can inhibit acidification of the phagosome. To investigate this possibility, we first attempted to use Lysotracker, an acidotrophic fluorescent dye which flows

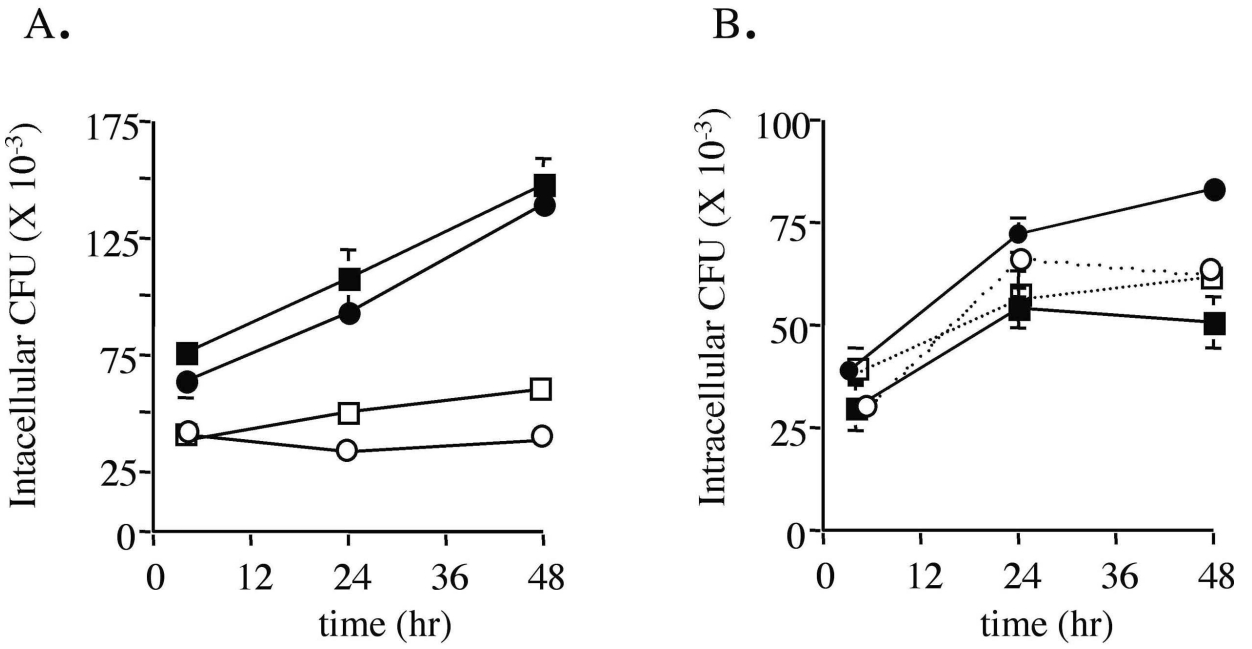

Figure 27. *M. tuberculosis* growth within activated DC and macrophage from RNI-deficient mice.

Cell pellet lysates of *M.tuberculosis*-infected DC (squares) and MØ (circles) (MOI=1) isolated from (**A**) C57Bl/6 mice or (**B**) NOS2-/- mice (kindly provided by Dr. Timothy Billiar), were unactivated (filled symbols) or activated (open symbols) with IFN-γ + LPS, then serially diluted in PBS plus 0.05% Tween 80 and plated on 7H10 plates, which were incubated for 18 days at 37°C and 5% CO_2. Three wells per condition were assessed at each timepoint, and the mean intracellular CFU values are reported at various time points after infection DC (squares) and MØ (circles). At the input timepoint <1% of total CFU was found in the supernatant. Representative of three experiments is shown. Error bars show standard error.

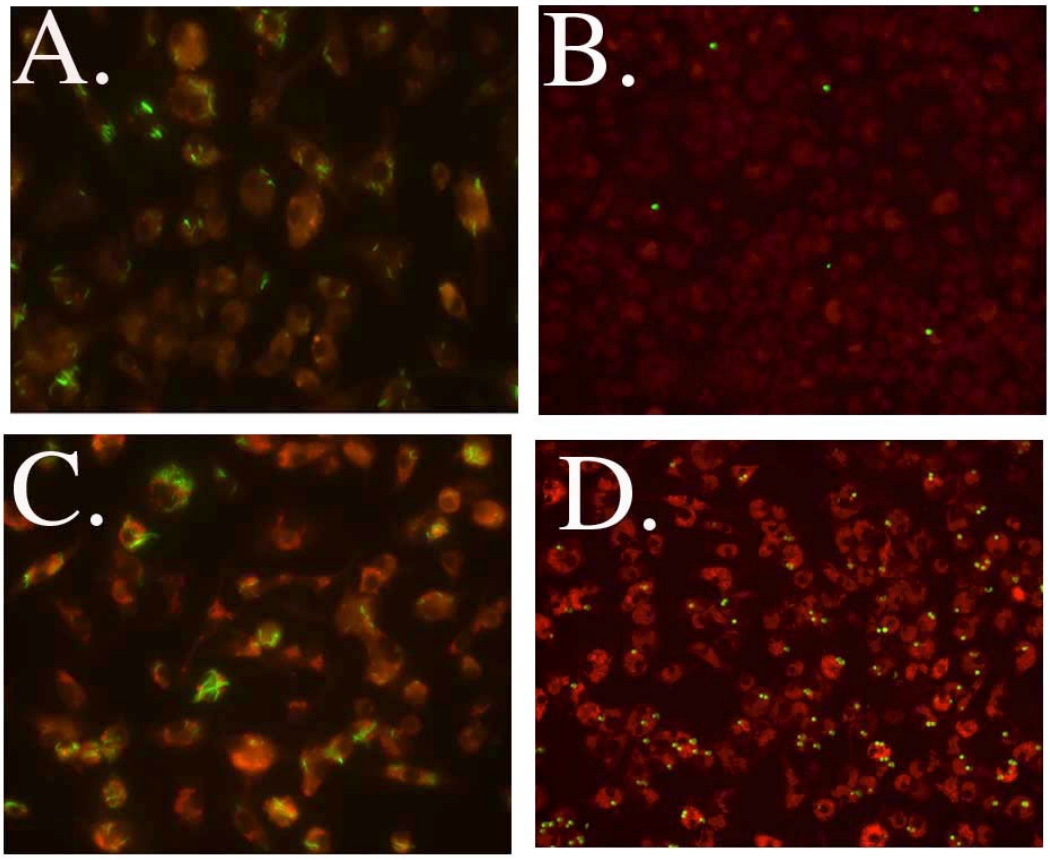

Figure 28. Lysotracker staining in unactivated and activated macrophages.

Bone-marrow derived macrophages were left untreated (**A**, **B**) or pretreated with Lysotracker Red (**C**, **D**) (Molecular Probes, Eugene, OR) then infected with GFP-labeled *M. tuberculosis* (green) (**A**, **C**) or given 3 micron Fitc-labeled beads (Polysciences, Warrington, PA) (**B**,**D**), washed 24 hours post-infection, fixed, and imaged with confocal microscopy. A representative field and experiment is shown. Repeated at least 10 times. Magnification 100X (A, C) and 60 X (B, D).

freely into the vacuoles and compartments of cells but is retained in acidified vacuoles and compartments due to protonatation of the dye, causing acidic compartments to fluoresce (Molecular Probes,Eugene, OR). The intensity of the dye is proportionately related to the pH (the more fluorescence, the lower the pH). A strain of *M. tuberculosis* that expresses Green Fluorescent Protein (GFP) was used in these experiments. We activated DC and macrophages as described previously, incubated with Lysotracker Red, infected with GFP-*M. tuberculosis,* and viewed cells at several timepoints ranging from 1 hr to 96 hrs post-infection using confocal microscopy (Figure 28, 24 hr pi). We used untreated and uninfected cells as our negative control (not shown). This control determines the level of autofluorescent background, which can be very high in phagocytes even in the rhodamine wavelengths. Since our cells were infected with virulent *M. tuberculosis*, they must be fixed prior to viewing. The level of autofluorescence in cells without lysotracker was very high and appeared punctuate, which appears as positive staining for lysosomes thus hampering our ability to determine actual pH staining (Figure 28 A, C). Even after several protocol changes, troubleshooting, and various microscopes, such as epifluorescent and live cell (with fixed cells), we were unable to eliminate background and identify real staining and hence unable to compare conditions. Using a technique based on Grating Imaging Systems for Optic Sectioning (233) we identified actual staining by recreating an image without the background (data not shown), but this technique can only recreate one cell at a time, and is very time-consuming, making it unrealistic for our studies.

As a positive control, macrophages were incubated with 3 micron FITC-labeled latex beads. Macrophages phagocytose these beads, then proceed through the endocytic pathways to fuse with the lysosome (28), and therefore lyostracker staining co-localizes with the beads and serve as a positive control. However, even in this control scattered staining not associated with

beads was observed, as well as in cells without beads (Figure 28D). In summary, this was not a clean enough system is to detect differences in pH, and probably would be completely useless for detecting subtle pH differences (as seen in Figure 28), as might be the case between DC and macrophages. We hope to eliminate problems with background autofluorescence by using live cell microscopy with other non-P3 level mycobacteria. There is an additional reason why this technique may not workin our system for measuring pH. *M. tuberculosis* resides in phagosomes which are tightly apposed to the bacilli; this does not allow for much fluid within the vacuole and may alter the effects of this dye and contribute to the problems with this technique in our system.

Δicl-*M. tuberculosis*- growth within activated DC and macrophage.

As an indirect method for testing the hypothesis that defective acidification of vacuoles could be partially responsible for the inability of DC to kill intrac*ellular M. tuberculosis*, we used a mutant strain of *M. tuberculosis*, which has a targeted knockout in the gene for isocitrate lyase (Δ-icl). This strain does not survive well in macrophages activated with a low dose of IFN-γ, which causes acidification but does not trigger production of NO (229). The intracellular growth of Δicl-*M. tuberculosis* within low dose IFN-γ-activated DC and macrophages was compared to the growth of wild-type *M. tuberculosis.* There was a decrease in number of surviving Δicl-*M. tuberculosis* in activated cells, compared to the wild-type strain. The decrease in Δicl-*M. tuberculosis* viability was much higher in activated macrophages compared to activated DC. This supports that hypothesis that DC may not acidify their phagosome/phagolysosomes to the same degree as

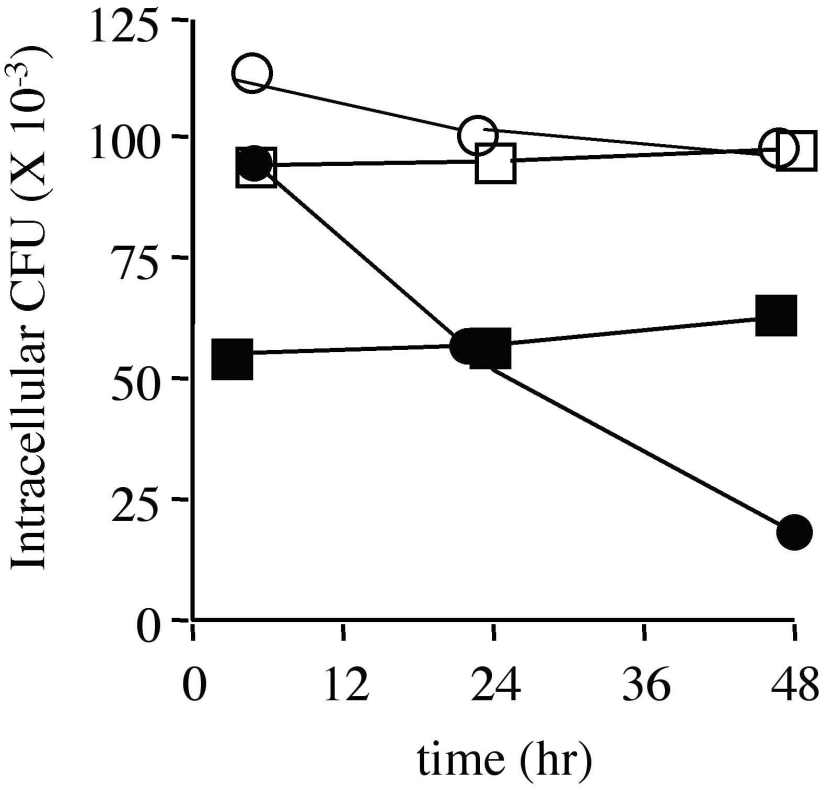

Figure 29. Δicl-*M. tuberculosis*- growth within activated DC and macrophage.
Cell pellet lysates of Δicl-*M.tuberculosis*-infected (filled) or *M.tuberculosis*-(open) low dose (50 units) IFN-γ-activated DC (squares) and MØ (circles) (MOI=1), were serially diluted in PBS plus 0.05% Tween 80 and plated on 7H10 plates, which were incubated for 18 days at 37°C and 5% CO_2. Three wells per condition were assessed at each timepoint, and the mean intracellular CFU values are reported at various time points after infection DC (squares), MØ (circles), Δicl-*M.tuberculosis,* (filled), *M.tuberculosis* (open). At the input timepoint <1% of total CFU was found in the supernatant. A representative of three experiments is shown.

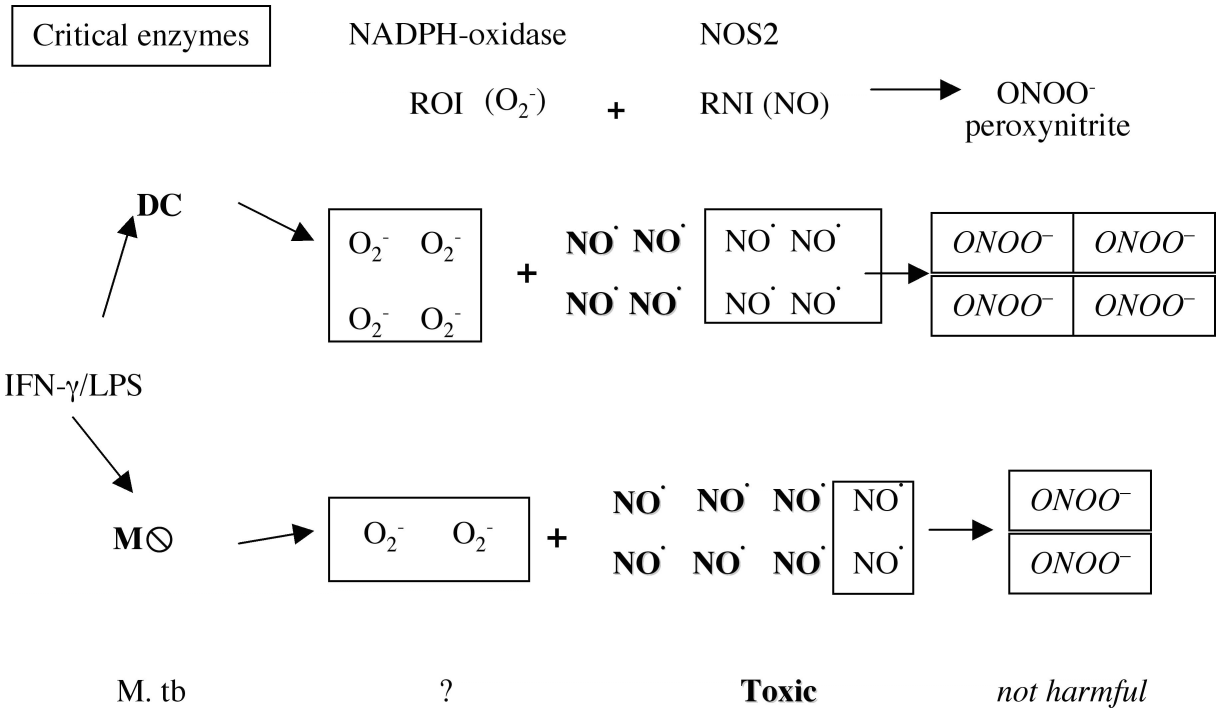

Figure 30. Theoretical model for anti-microbial compound production in activated DC and macrophages.

This diagram depicts a very oversimplified view to how the activation of DC and macrophages can lead to differential production of peroxynitrite which does not kill *M. tuberculosis*, and leave different quantities of toxic nitric oxide behind which can kill. We hypothesize that these ratios are important for differences in killing between these cells.

Note this model excludes all other ROI and RNI production for simplicity

activated macrophages, since they cannot as efficiently kill Δicl-*M. tuberculosis,* which is very sensitive to acidfication.

Discussion

DC are most commonly thought of as APC. Indeed, *M. tuberculosis*-infected DC are better APC than infected MØ, in terms of priming and restimulation of T cell responses (23), (97), (14), (124), (data not shown). Recently CD40-CD40L interaction was shown to be a potent stimulus for DC in response to a mycobacterial infection (14). DC are capable of initiating a protective immune response to *M. tuberculosis* since upon infection they contribute important components of immunity, such as inflammatory cytokine production and T cell interaction (14). We investigated whether DC also acted as an effector cell, and therefore could possibly contribute to control of the infection and participate in the direct killing of *M. tuberculosis*.

DC have been demonstrated to take up and harbor various microbes, including *Salmonella*(160, 187), *E. coli* (187), *Listeria monocytogenes* (188), *Borrelia burgdorferi* (161, 189), *Bordetella bronchiseptica* (159), BCG (158, 190), *Leishmania* (162, 163, 191), *Chlamydia* (164, 165, 192, 193), *Candida albicans* (195), and *Histoplasma capsulatum* (194). DC have been shown to directly kill *Chlamydia trachomatis* and *Chlamydia psittaci* by phagosome-lysosome fusion and this led to priming of *Chlamydia*-specific T cells (165). DC have been shown to phagocytose *Borrelia burgdorferi*, which was later detected in the phagolysosome, then processed and loaded on MHC class II molecules (161). As well *Histoplamsa capsulatum* (194) and *Candida albicans* (195) are phagocytosed, killed by phagolysosome fusion, processed, and presented to T cells by human DC. Interestingly, *Histoplamsa capsulatum* prevents phagolysosome in human macrophages but not in DC. *Candida albicans* caused a large

reduction of phagolysosome fusion in the DC, as compared to *Histoplamsa capsulatum*, but hydrolases within the phagolysosome are ultimately hypothesized to kill the fungus, *C. albicans* was shown to trigger ROI production but this did not contribute to the killing of this organism (195). Other anti-microbial mechanism of activated DC have not been investigated.

We first investigated RNI production since it is known that murine macrophages use this mechanism to kill intracellular *M. tuberculosis* (61), (50), (52). Murine DC have been shown to produce NO (55). Thymic DC generate NO in response to self and allo-antigens and kill auto-reactive T cells (203), including in the Experimental Allergic Encephalomyelitis (EAE) mouse model for Multiple Sclerosis (54), (53). We reported previously that activated DC produce RNI and can prevent *M. tuberculosis* intracellular growth yet they do not appear to kill the bacilli (23) (Chapter2). Despite these differences in killing there is comparable nitrite production by both activated DC and macrophages throughout 72 hrs post-infection (23). We sometimes observed a difference in nitrite production early after infection (4hr). The activated *M. tuberculosis*-infected DC actually had higher levels of NOS2 mRNA and protein but this did not result in higher nitrite production; regardless of the output of nitrite at 4 hrs the activated DC did not kill the intracellular bacteria. Possibly DC may regulate the activity of the NOS2 enzyme post-translationally, which inhibits NOS2 to prevent high levels of RNI output, or has a different availability of substrates for NOS2 (such as arginine).

ROI and peroxynitrite following interaction with NO are potent oxidative compounds which are toxic to many pathogens such as *Rhodococcus equi* (218), *Escherichia coli* (219), *Salmonella enterica* (68), *Salmonella typhimurium* (220), *Mycoplasma pulmonis* (221), and *Candida albicans* (222). The role of ROI, as discussed before, in *M. tuberculosis* infection is controversial, and so is the role for peroxynitrite. Indeed although it was speculated that

peroxynitrite could kill *M. tuberculosis* it has never been demonstrated. In fact, the opposite has been reported: virulent strains of *M. tuberculosis,* such as the most prevalent New York clinical strain, Strain C, and the laboratory strain Erdman, are highly resistant to peroxynitrite. (49). When we investigated the production of these compounds from activated DC, surprisingly DC made more ROI than macrophages as well as peroxynitrite. The production of peroxynitrite is dependent on superanion, (O_2^-) which is formed via the NADPH-oxidase complex. Mice which had a genetic disruption of the $p91^{phox-/-}$ subunit of this complex can no longer produce ROI and we show that this abrogates their ability to produce peroxynitrite as well (Figure 26A).

Based on these data, we hypothesize that since activated DC make more peroxynitrite than activated macrophages, they are less efficient at killing *M. tuberculosis*, because the amount of effective RNI (such as NO) is diminished, due to the higher ROI levels, which pull away NO molecules to form peroxynitrite (Figure 30). *Leishmania major* is susceptible to killing by NO production within macrophages but is resistant to both ROI and $ONOO^-$ killing (223). We have also shown that activated DC can kill a non-pathogenic species, *Mycobacterium smegmatis,* and an a virulent strain of mycobacteria, BCG, which have been reported to be susceptible to peroxynitrite (Figure 23 & 24).

In order for *M. tuberculosis* to survive in the lung they probably need defense mechanism against peroxynitrite (221). Ruan, et. al. found a gene in *M. tuberculosis*, noxR3, postulated to protect the bacteria from RNI and ROI. When noxR3 from *M. tuberculosis* was expressed in *Salmonella typhimurium* it conferred resistance to these both oxidative and nitrosylative stress (233). In addition, the lethal effects of NO can be modified. *M. tuberculosis* has been shown to have peroxynitrite reductases, peroxiredoxins, which reduce the oxidizing and toxic effects of peroxynitrite (234). These peroxidoxins's reductase activity is based on the reversible oxidation

of their N-terminal Cys, therefore under conditions of chronic exposure or very high concentration of peroxynitrite their reductases activity is hindered since the active sites of Cys will remain oxidized (234). In the presence of small amounts of peroxynitrite or fluxes of production, possibly in acute infection, is probably when the peroxidoxins can work best. Interestingly, it was shown that small amounts of peroxynitrite may participate in signaling cascades which actually help the cell activate enzymes that repair tyrosine nitration (234). It has been reported that surfactant enhances peroxynitrite production possibly via SP-A, a surfactant protein, which has been suggested to mediate ROI release in the lungs (221) It is likely that a defense mechanism against peroxynitrite is necessary for *M. tuberculosis* to survive long term in the lung. These could explain why we were unable to detect the presence of nitrosylated proteins. It was reported that an excess of ROI production may convert NO into NO_3^- by dioxygenase, which is inert and not anti-microbial (235). Activated DC are capable of making more ROI therefore they in turn may make more NO_3 and alter the lethal effects of their pool of RNI.

We hypothesized that the excess production of ROI by activated DC leads more $ONOO^-$ and less tuberculocidial RNI, such as NO, contributing to the inability of DC to kill *M. tuberculosis.* To address this further, the growth and killing of *M. tuberculosis* within p91 $^{phox-/-}$ murine macrophages, which cannot produce ROI, and NOS2-/- macrophages which cannot make NO were tested. Neither mouse should be capable of peroxynitrite production since this is dependent upon 0_2 and NO (236). We confirmed that p91 $^{phox-/-}$ did not produce significant levels of peroxynitrite as compared to WT cells (Figure. 26). It was reported that p47 $^{phox-/-}$ mice are subtly impaired in the initial control of *M. tuberculosis* infection (216). We report here that the activated DC from the p91 $^{phox-/-}$ mice could reduce the number of viable intracellular bacilli

below the input number (figure 25A), in contrast to activated DC from wild-type mice (Figure 25B). However, the activated macrophages from p91 $^{phox-/-}$ mice could not reduce the number of viable intracellular bacilli below input and even allowed for a small amount of bacterial growth; this differed from wild-type macrophages which can routinely kill 50% of intracellular *M. tuberculosis* (Figure25). We hypothesize that p 91$^{phox-/-}$ DC can kill the intracellular *M. tuberculosis* because the NO needed for killing is not converted to peroxynitrite due to the relative absence of ROIs. However, it appears macrophages use ROI in some capacity to make NO efficient at killing, which we believe is not the formation of peroxynitrite. Roles for ROI in signaling within macrophages may lend to killing via cytokine production or undiscovered mechanisms that may contribute to killing of *M. tuberculosis* (216). As well ROI themselves may be able to kill, it has been reported that *M. tuberculosis in vitro* was susceptible to hydrogen peroxide killing (47). ROI have been implicated in non-phagocytic cells to contribute to signal transduction pathways which lead to apoptosis (237).

Further support for the hypothesis is that ROI may contribute to killing within activated macrophages and not DC came from examining the growth of intracellular *M. tuberculosis* within NOS2-/- bone-marrow derived macrophages and DC (Figure 27). In these cells the ability to kill *M. tuberculosis* is negated due to the inability to produce NO or RNI, but the activated macrophages do appear to slightly reduce the number of colonies below that of the unactivated (which do not produce ROI). However in the NOS2-/- bone-marrow derived activated DC the growth of intracellular *M. tuberculosis* is either the same as or more than the unactivated cells which indicates that the ability to hinder the growth is completely abrogated. The only time the activated DC were capable of killing intracellular *M. tuberculosis* was under conditions where ROI were drastically diminished but NO was present as seen in the p91 $^{phox-/-}$

mice. Studies have shown that when ROI are depleted by superoxide dismutase there is a higher production of RNI. We did not observe this; there was a comparable level of RNI in wild-type and p91 $^{phox-/-}$ macrophages following activation. Thus, differences in killing may be attributed to differences in the availability of NO.

M. tuberculosis can be killed in the acidic environment of a phagosome (238). It was shown that *Coxiella burnetti*-containing phagosomes, which have a low pH, fused with *M. tuberculosis* containing phagosome thereby lowering the pH and killing the mycobacteria (201). Mycobacteria have been shown to prevent this acidification (239). *M. avium* and *M. tuberculosis* can prevent the acidification of the phagosome by excluding ATPase pumps (30), (31). *M.tuberculosis*-containing phagosomes exclude Rab7, a late endosomal GTPase protein, thus indicating that these phagosomes did not fuse with the lysosome and acidify the phagosome (33). Even if *M. tuberculosis* containing-phagosomes express Rab 7 they still do not fuse with the lysosome suggesting interference with the function of Rab 7 (240). In an activated macrophage fusion of the *M .tuberculosis*-containing phagosome to the lysosome is much more frequent, presumably increasing the amount of killing. Therefore, if the activated DC is impaired in the ability to fuse with lysosomes and/or acidify the phagosome, then this would lead to an inability to kill. DC infected with *C. albicans* showed a seventy percent reduction of phagolysosome fusion, as compared to *H. capsulatum* infection of these cells, showing that DC can regulate the level of phagolysosome fusion during infections (195). Acidification can also contribute to the potency and increase the half-live of NO, $ONNO^-$, and ROI so a deficiency in acidification could reduce toxic effects of these compounds (56). The ability of macrophages and DC to acidify the *M. tuberculosis*-containing phagosome was compared.

We first tried to assess acidification by a direct method, using a Lysotracker dye, but due to problems with high level of background autofluorescence we were unable to confidently assess acidification in activated DC and macrophages. We had the same problems using an antibody against LAMP, a lysosomal marker, to try to determine if there were differences in lysosomal fusion with phagosomes-containing *M. tuberculosis* in activated DC compared to activated macrophages (data not shown). The difficulties with Lysotracker dye and direct assessment of acidification led us to approach this question by an indirect method. We compared the survival of a strain of *M. tuberculosis* with a mutation in the gene for isocitrate lyase (*icl*) in DC and macrophages. Isocitrite lyase is an important enzyme in the glyoxylate shunt in bacteria; when genetically disrupted in *M. tuberculosis*, Δicl, the bacteria was reduced in persistence in mice and has much decreased viability in acidified macrophages (229). In these experiments, the macrophages were activated with a low dose of IFN-γ and no second signal for NOS2; this resulted in fusion of phagosomes with lysosomes and subsequent acidification, but no RNI production. We compared the growth of Δicl *M. tuberculosis* in IFN-γ-activated DC and macrophages to determine whether differences in the intracellular environment of the activation states of these cells results in differential viabilities of this acid-sensitive strain. The growth of Δicl *M. tuberculosis* in low dose activated cells is much reduced below WT *tuberculosis* or Δicl *M. tuberculosis* growth in unactivated cells. Interestingly, in low IFN-γ dose activated DC there was much less killing of Δicl *M. tuberculosis* as compared to macrophages (Figure 29). This suggests that the activation state of the DC is different and possibly not as acidic as the macrophages. Future plans include using immuno-EM with an antibody against DAMP, a molecule which accumulates in acidic compartments, on infected activated DC and macrophages given the DAMP, to quantitate difference in acidity between cells.

In vivo, only a small percentage of DC or macrophages are activated at any one time. *M. tuberculosis* likely grows within the unactivated cells, and thus the infection is not cleared. Even within the activated state of a macrophage, only half the tubercle bacilli are killed, at *least in vitro*, and our data indicate that within a DC, killing does not occur. *M. tuberculosis* manages to live within these hostile environment, yet the elimination of some portion of the population *of M. tuberculosis* is critical for the host-pathogen balance that is generally observed, i.e. latent infection. We propose, based on the data presented here, that the role of the activated DC in maintaining a mycobacteriostatic, but not mycobactericidal environment may be crucial for protection, as it may enhance their potent antigen presenting properties. Presentation of antigen from living microbes is more efficient than from killed organisms, and live *M. tuberculosis* matures DC better than killed bacilli (22). This has been shown for *Leishmania major* and speculated by others to contribute to long-term immunity against an organism (163), (155).

Early in infection, the DC may also contribute to protection by halting uncontrolled growth of the bacilli. These data show that there are differences in the vacuolar environment between activated DC and macrophages, and this may contribute to an inability to kill *M. tuberculosis.* Our data also support that peroxynitrite does not kill *M. tuberculosis*. However, ROI may play a role early in activated macrophages to help kill the intracellular microbes. In an odd way ROI may contribute to protection in DC by allowing these cells to maintain viable bacteria. The differences in acidification of the vacuoles could lead to differences in how antigen is processed, loaded, and presented. Low acidification, as we suspect in the DC, may be more beneficial for antigen presenting, whereas low pH favors killing of bacteria, as seen with macrophages. We believe that DC play a distinct role in initiating the protective immune

response to *M. tuberculosis*, and the unique properties of this cell with respect to *M. tuberculosis*, described in this work, contribute to this function.

Summary

Mycobacterium tuberculosis is responsible for the largest number of infections from a single organism, and is only second to HIV in the number of deaths per year from an infectious agent (1). This high number of deaths occur despite effective antibiotic treatment, widespread vaccination with BCG, and the host's ability to mount a protective immune response. It is estimated that only one of every ten immunocompetent people infected with *M. tuberculosis* develops active tuberculosis. Numerous studies over the year have shown that cell-mediated immunity is necessary for protection (reviewed in (27)). Macrophages are the host cell for the bacilli and upon cytokine-induced activation kill *M. tuberculosis*. Although, macrophages are APCs they have been shown to be suppressive in the lung (241). Alveolar macrophages are inefficient APCs (242). DC are potent APC and generate immunity in the airways after infection to a much larger extent than macrophages (140). In this study we sought the effects of DC interacting with *M. tuberculosis*, both on the DC and on the intracellular bacteria.

We first examined whether human peripheral blood derived DC and murine bone-marrow derived DC could be infected with *M. tuberculosis*. Macrophages were previously the only known reservoir for this bacterium. We determined that DC were readily infected with *M. tuberculosis*; and there was a comparable percentage of DC infected as compared to macrophages in an *in vitro* system. The bacteria grow as rapidly within unactivated DC as macrophages (Chapter 1). Thus DC may provide another repository for the bacteria in the host. Despite similarities in infection, these two cells responded differently to the bacteria (Chapter 1 & 2). DC downregulated their phagocytic ability and upregulated molecules important for antigen presentation and trafficking, such as B72, MHC I & II, and ICAM. This is a hallmark of DC development in which they shift from an antigen processing phenotype to antigen presenting phenotype in response to bacterial stimulus (186). Macrophages expressed these markers but

they did not change significantly after infection nor did their phagocytic ability decrease after infection (Chapter 1). Functionally we showed that *M. tuberculosis*-infected DC were better than macrophages at stimulating T cells from infected mice. These studies suggested that DC play a role in initiating immunity (Chapter 1). There have subsequently been reports in the literature to support this (14).

DC in the lungs of mice have been shown to be infected (146) but it is still not known whether they are present in the granuloma or play a role in its formation. DC secrete IL-12 in response to *M. tuberculosis*-infection and in other systems this is boosted upon interaction with T cells (151), (182). IL-12 is important for the generation of a TH-1 T cell response that is necessary for protection in a *M. tuberculosis* infection. In addition DC secrete inflammatory cytokines which are important for cell migration and granuloma formation (23), (147), (166). DC secrete TNF-α which is a crucial cytokine in activation of macrophage, inducing the inflammatory response, migration of cells, and organized granuloma formation (232). DC also stimulate T cells to secrete IFN-γ which is a crucial cytokine in an *M. tuberculosis* infection. Mice deficient in this cytokine succumb to tuberculosis faster than any other single gene deficient mouse. IFN- γ is necessary with a second signal to activate macrophages and DC (23) (Chapter 1). These data show that DC produce protective immune responses and are better APC in the infection. We believe these data support our hypothesis that DC in lung that are infected, mature, then traffic to the draining lymph node to prime naïve T cell against mycobacterial antigens.

In addition to characterizing what happens to the DC after infection we examined the fate what of *M. tuberculosis* in the intracellular environment, especially after activation with IFN- γ and LPS (Chapter 2 & 3). There has not been much research in DC for anti-microbial

mechanism or compounds, with the exception of antigen processing mechanisms, such as late endosomal trafficking and fusion of the phagosome to the lysosome. The production of such compounds may be necessary for protection and viability of the DC during infection, and the DC may limit harmful bacterial burdens in infections, such as *M. tuberculosis*. Activation of macrophages by IFN-γ and a second signal leads to the production of NOS2 generated RNI, NADPH-catalyzed ROI, and phagolysosome fusion and acidification (reviewed in (27)). We know that RNI present in murine macrophages can kill *M. tuberculosis.* The role for ROI in *M. tuberculosis* is debatable but its production by macrophages is known to play a role in many infections, including *M. microti* (12). The production of ROI was never investigated in DC. Although acidification has been shown to kill *M. tuberculosis*, it is questionable how often this happens *in vivo* since the mycobacteria can prevent acidification. Macrophage activation allows endosomal procession to fusion with the lysosome, therefore under this circumstance some killing of *M. tuberculosis* would occur (33). *In vivo* we know that not all cells are activated and those that are have a limited lifespan and may eventually die. The remaining intracellular *M. tuberculosis* may be taken up by a unactivated macrophage, which will allow for its growth. Neither RNI production and/or acidification in the activated macrophage can clear this infection but they are important mechanisms in the stand-off between the bacterium and the host.

We have found these anti-microbial mechanisms present in IFN-γ and LPS activated murine DC but we see a different outcome for the bacteria (Chapter 3). This suggests that DC have an activated state that is different from the matured state. We were not surprised to find that DC produce RNI since this had been reported previously (55), but despite an equal production of nitrites by the infected-activated DC, they only hinder the growth of *M. tuberculosis* and do not kill it. In addition the activated DC produced a larger quantity of ROI

which leads to a higher production of peroxynitrite (Chapter 3), since ROI is the limiting factor in peroxynitrite formation from NO and O_2^-. In spite of more of these compounds the activated DC cannot kill intracellular *M. tuberculosis,* but can kill *M. smegmatis* and BCG which are non-pathogenic (Chapter 3). Several species of bacteria and parasites are resistant to peroxynitrite killing, including *M. tuberculosis* and *Leishmania major*. Protection to *Leishmania major* in mice has many similarities that of *M. tuberculosis* including the necessity of a TH1 response which is driving by the DC. *Leishmania major* is also not killed within DC but remains viable while the DC traffic to the lymph node and was even found to remain viable within DC in the lymph node up to several days post-infection (162, 163). Virulent strains of *M. tuberculosis* were found to be amazingly resistant to very high doses of peroxynitrite but susceptible to nitric oxide and nitrogen dioxide. Avirulent strains were susceptible to peroxynitrite killing as well as nitrogen dioxide and nitric oxide killing (49). Recent data has shown that peroxredoxins are present in cells, including bacteria such as *M. tuberculosis*, and prevent destructive oxidation, especially under low concentrations of peroxynitrite (234). This could explain our lack of detection of nitrosylated proteins within *M. tuberculosis*-infected DC and macrophages (Chapter 3). Nitrosylation of protein occurs due to the presence of peroxynitrite (68). If *M. tuberculosis* can reduce the peroxynitrite then this would alleviate cell damage and nitroyslation. In addition these reductases may be present and active in DC and macrophages.

Our hypothesis, derived from these data, is that the higher quantity of ROI from DC utilizes the available NO to form peroxynitrite which is not toxic to this bacteria. Therefore the DC uses up NO which is lethal for *M. tuberculosis* to produce a compound ($OONO^-$) which which is not. It is unclear whether the bacteria or DC adapted this strategy for its benefit. We have tested this hypothesis by examining growth of *M. tuberculosis* within mice deficient in ROI

production, $p91^{phox-/-}$, in which there are still RNI present but no peroxynitrite since it needs superanion to be produced. We determined that DC from $p91^{phox-/-}$ mice were able to kill intracellular *M. tuberculosis* (Chapter 3). This is probably because RNI are available and not sequestered by production of peroxynitrite. In mice which lack NOS2 and RNI production, DC were incapable of inhibiting the growth of intracellular *M. tuberculosis*, in contrast to DC from wild-type mice. However, activated macrophages from NOS2 mice appeared to allow for a slight amount of *M. tuberculosis* growth suggesting the ability of macrophages to kill *M. tuberculosis* involves ROI (Chapter 3). We believe this is not dependent on the formation of peroxynitrite since this compound does not kill *M. tuberculosis*. It is possible that another compound is formed with some species of ROI and RNI within activated macrophages, and this may not occur in activated DCs. ROI is involved in important signaling mechanisms within the macrophages (216) that may turn on a NOS2 independent mechanism for killing or simply turn on the production of certain cytokines such as TNF-α. We did not examine cytokine production from these infected knock-outs. Data have shown roles for ROI-mediated signaling in cytokine production and in cell trafficking (216). NOS2-independent mechanisms are believed to exist and not yet identified in activated macrophages.

Importantly a reduction in acidification in the phagosomes of DC could greatly affect their ability to kill. Studies have shown that phagolysosome fusion occurs in DC. This has been shown to kill *Chlamydia* species and *Borrelia burgdorferi* has been identified in the phagolysosome then processed and presented on MHC class II (165), (161). It is known that antigens which are presented by MHC class II must proceed through to the late endosomal compartments so that they can fuse to class II containing compartments (243). Therefore DC possess to ability to kill microbes for presentation by acidification via the lysosome or class II

compartment and this is the best studies anti-microbial mechanism in DC. The amount of acidification has never been directly compared to that of a macrophage. We have data suggesting the activated DC may not have as acidic of a phagosome environment. A mutant strain of *M. tuberculosis*, Δicl, has been shown to be sensitive to killing within the acidified state of a macrophage, which is achieved by low dose IFN- γ activation (229). Therefore indirectly, this mutant is an indicator of pH within the phagosome. Δicl *M. tuberculosis* is susceptible to killing within an low dose IFN- γ activated DC, whereas the wild-type strain is not and the Δ icl M. *tuberculosis* is not killed within unactivated DC. However, the low dose IFN- γ activated DC does not come close to killing as much Δicl M. *tuberculosis* as the activated macrophage can. This suggests that there is an acidification difference between these activated cells. It confirms what our other data has shown that the activation state of these cells is different. This difference in pH could limit the toxicity of anti-microbial compounds such as ROI, NO, and peroxynitrite and hinder their ability to kill. The killing effect of these compounds on microbes is usually determined from activated macrophages where the pH is very low. *In vitro* assays have shown that low pH increase the half-lives of these compounds (68) and this could contribute to difference in killing. As well it may be of interest to investigate gene expression of *M. tuberculosis* within activated DC and macrophages, as these environment are different there may be unique gene expression within the bacteria.

We believe that the bacteriostatic effects of the DC contribute to their ability to be a potent APC in this infection. It has long been known that live *M. tuberculosis* and presumably secreted antigens from this bacilli can generate more immunity than dead bacilli (133), (12). To sustain an immune response to chronic infection such as *M. tuberculosis* and *Leishmania major*, it is believed that the DC would have to harbor live antigens which could continually be secreted

(163), (155). These studies help establish that DC are important in the initiation of an immune response. They also identify DC as being effector cells and preventing bacterial growth as well as producing compounds which can kill microbes. This research will lead to further investigation of the differences between the activated states of DC and macrophages, as well as elucidate the different role these cells play in infection. Understanding DC and how they process mycobacterial antigen and generate immunity will be very beneficial toward understand how to protect the host during infection.

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